NEUROPLASTICITY AND NEUROTOXICOLOGY:

CENTRAL BREATHING CONTROL FOLLOWING DEVELOPMENTAL

NICOTINE OR ETHANOL EXPOSURE

By

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April 20, 2010

Date

NEUROPLASTICITY AND NEUROTOXICOLOGY: CENTRAL BREATHING CONTROL FOLLOWING DEVELOPMENTAL NICOTINE OR ETHANOL EXPOSURE

A

DISSERTATION

Presented to the Faculty

of the University of Alaska Fairbanks

in Partial Fulfillment of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

By

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May 2010

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ABSTRACT

Nicotine or ethanol exposure early in development are both risk factors for Sudden Infant Death Syndrome (SIDS). I tested the hypothesis that both nicotine and ethanol may be linked to SIDS by impairing central breathing control responses to low oxygen (hypoxia) and high carbon dioxide (hypercapnia) stressors. Experiments were conducted in bullfrog tadpoles, a model system for respiratory neurotoxicology research. I addressed three specific aims: to characterize the effect of chronic ethanol on central responses to hypercapnia and hypoxia, to characterize the effect of chronic nicotine on central hypoxic responses, and to determine the persistence of hypercapnic impairments following 10-wk exposure to either nicotine or ethanol. 10-wk nicotine exposure resulted in neuroplastic changes that eliminated the central hypoxic responses of early but not late metamorphic tadpoles. Thus, central responses to both hypoxia and hypercapnia were impaired following nicotine exposure. The attenuated central hypercapnic response of nicotine-exposed tadpoles persisted for 1 - 3 wk. Following 10-wk chronic ethanol exposure central responses to hypercapnia and hypoxia were lost regardless of the developmental timing of exposure. Impairments in central hypercapnic responses persisted for 3 - 6 wk after ethanol exposure ended. The

recovery of central hypercapnic responses in nicotine- and ethanol-exposed tadpoles may be an example of recuperative neuroplasticity resulting in either a reinstatement of network components and functions or an accommodation to deleterious nicotine- and ethanol-evoked neuroplastic changes. Collectively these data suggest that both nicotine and ethanol may target adaptive and compensatory mechanisms in central breathing control. The teratogen-induced impairments were developmentally dependent in the case of nicotine, and they persisted longer following ethanol exposure. The overall result of exposure to either neuroteratogen was an inability to respond to central breathing stressors, supporting the possible link to SIDS.

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ACKNOWLEDGEMENTS

Dr. Barbara Taylor. If it wasn't for your rash decision to take a chance on an over-zealous kid from Colorado, we would be in very different places right now. Your leadership has given me the opportunity to develop my skills as a scientist, mentor, and independent thinker. Thank you for your support and continued willingness to provide me with any and all of the resources I need to succeed. You have the amazing ability to think critically at the heart of any issue, you taught me to look for the "emperor's clothes" in a world of naked emperors. I am forever in your debt.

Dr. Michael Harris. My acceptance to veterinary school in Colorado was by far the proudest achievement of my life, one that I know would not have been possible without your efforts. Your brilliant scientific mind and unique perspective were always quick to my aid. Thank you for your help making my dreams a reality.

Dr. Jonathan Runstadler. Thank you for being a sounding board, mentor, and advocate. You have been my confidant, counselor, and friend. I've appreciated the perspective you have brought to my research. I look forward to being your colleague in the future.

Dr. Kelly Drew. The position I had in your lab was the first in what I hope will be a long line of research positions. You have taught me what it takes to be a competitive scientist and have always kept me striving to do more. Thank you for your continued encouragement.

Justin Buehner, Spencer Audie, Megan Hoffman. I was fortunate to share a lab with three very different thinkers. I am honored to have worked with you. Our opinions and perspectives often put us at odds with each other, but perhaps like siblings we knew we could still count on one another. I have the deepest respect and admiration for each of you. Your insight was invaluable to me. I know you will accomplish all that you set forth to do and more.

Brittney Dykstra Davies and Jessica Lamb. I was fresh into graduate school when you two took a chance and let me serve as your mentor for projects I had no familiarity with. We learned together and matured as young investigators. I made many mistakes, but came out a more equipped and resourceful instructor after our time together. Thank you for all that you taught me about myself. I hope you remember my intentions more than my skill level if ever I am your medical patient.

Carla Cartagena, Nicholas Bajada, Lisa McLane, Carla Nelson, Emily Potter. I would still be revising manuscripts from my Master's if it wasn't for your help. I imagine many grad students leave their lab with a list of "what-ifs" and "if-I-did-it-over-agains". Your projects have given me a chance to explore untested avenues and aspects of our field, places I would never have been able to go without your help. I regret not having more time to spend with each of you. I look forward to hearing of your many successes in the years to come.

Kimberly Iceman, Trixie Lee, Melanie Richter. Having you each rotate through the lab was a pleasure. I wish more students had that opportunity; not only do I appreciate your help on my projects, but you have been my friends and sounding boards throughout our graduate career. I feel like we are classmates, and I wish we were all graduating together. Thank you for your support, do stay in touch.

Dr. Lena Vayndorf and Dr. Liel Rubinsky. Thank you for teaching me the ways of a post-doc. You were thrown into projects beyond your comfort zone; like mice in a water maze, your skills and versatility always kept your heads above water. You tolerated me, with grace and maturity. Thank you for your guidance and scientific prowess. I wish only the best for you in the future.

Hailey Chacon, Tiffany Chen, Sheila Spader, Kennan Jeannet. Our time working together in the lab was brief. I was thrilled with your energy and enthusiasm. It is hard saying good bye to a job that has been such a large part of my life the past few years. With eager students like you willing to step in, the future success of the lab is secured.

Students in the Harris Lab. It must have been hard not being in our lab. We had all of the fun. Sure you had all the neat toys, but those ended up in our lab anyway. It was great sharing the hall together. Thanks for your help on my projects and for the dry-erase boards whenever I needed to scratch my creative itch.

Fellow Biology Students and Friends. Finding a balance between research and reality was a huge struggle for me, made easier through our association. I am grateful for the camaraderie and fresh perspective on the future of biology. Next round's on me.

UAF Faculty. Before I came to UAF, I thought all faculty members lived in ivory towers. UAF has a refreshing assortment of skilled researchers who demonstrate competency without pomposity. I enrolled in enough classes for three Master degrees. Each course had an atmosphere of

mutual respect and collaboration. My success would not have been possible without such a welcoming and supportive learning environment.

Michael, Debra, Kye and Norah Brundage, Karen, Ramey and Jeffrey Wood, and the rest of my family. Words can not express what your love and encouragement means to me. You made huge sacrifices, and you were always willing to do more. Thank you for helping me remain true to myself.

Brumbi. Thanks for giving me a reason to go home at night.

Fairbanks, AK. It was nice knowing you.

National Institute of Health. My program was supported by the Alaska Basic Neuroscience Program, which is funded by a Specialized Neuroscience Research Program grant. My research was paid for by a National Institute of Health grant through the National Institute of Neurological Disorders and Stroke. Grant # 2U54NS041069-601A. I appreciate the support.

The Bullfrogs. At the time I wrote this 583 animals were sacrificed by my hand for this and my related research. All procedures outlined in this

thesis were approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee (IACUC; approval # 04-04; 07-04).

CHAPTER ONE

General introduction

Breathing is a vital behavior; it plays an essential role in the exchange of gases necessary to maintain life. The control of breathing, which must tightly regulate systemic conditions, must be fully functional at birth to orchestrate the first breath (Achard et al., 2005, Greer et al., 2006). Neural control of breathing has been the subject of considerable research, and there is much to be understood. There are several conditions in which the control of breathing falters in early development leading to notable pathologies and even death (Gaultier, 2000, 2001, Chen and Keens, 2004, Gozal, 2004, Abu-Shaweesh and Martin, 2008, Ogier and Katz, 2008). Understanding the drives that maintain breathing and pathologies that interfere with these drives will aid in the treatment, and ideally prevention, of breathing-related pathologies.

Ethanol and the active ingredient in tobacco smoke, nicotine, are both neuroteratogens (West and Goodlett, 1990, Seidler et al., 1994, Abreu-Villaca et al., 2003, Goodlett et al., 2005). Exposure to either nicotine or ethanol during development has been associated with numerous functional and neurological deficits (King and Fabro, 1983, Brien and

Smith, 1991, Dwyer et al., 2008). In recent years, the consequences of exposure to nicotine or ethanol on the control of breathing have grown as a research interest (Hafstrom et al., 2005, Dubois et al., 2006, Dubois et al., 2008a, b, Eugenin et al., 2008, Fregosi and Pilarski, 2008, Mahliere et al., 2008, Campos et al., 2009). Interest was sparked by epidemiological studies that determined infants born to mothers who smoked or drank during pregnancy had an increased risk of sudden infant death syndrome (SIDS; Milerad and Sundell, 1993, Iyasu et al., 2002, Kinney et al., 2003, Blair et al., 2006, Kinney, 2009).

Studies seeking to identify a causative link between nicotine or ethanol exposure and breathing disorders have had mixed results (Smith et al., 1991, Bamford et al., 1996, Watson et al., 1996, Eugenin et al., 2008). Difficulties arise in designing animal studies in species where the influences of maternal-fetal interactions can be differentiated from the influences of nicotine or ethanol (Hafstrom et al., 2005). It is also difficult to design studies that test the impact of nicotine or ethanol on the development of central breathing control because most of the experimental preparations established to test central breathing control have a limited developmental window of viability (Fong et al., 2008). Both

these difficulties can be overcome by using the bullfrog tadpole animal model.

Bullfrogs are oviparous; eggs are laid in water and development occurs as free-living aquatic tadpoles that metamorphose into frogs, a process that can take up to two years. Nicotine, ethanol, and other agents can be added directly to the water where the animals develop, and the concentration of these compounds can be easily monitored and manipulated (Taylor et al., 2008). Nicotine and ethanol both cross the epithelial layers of bullfrog skin and exposure can be highly controlled (Yorio and Bentley, 1976, Horimoto and Koyama, 1982, Hedin and Larsson, 1986). The central control of breathing in bullfrogs has been well characterized, and isolated brainstem preparations used to asses central breathing activity are viable and stable at all free-living bullfrog life stages (early metamorphic tadpoles - geriatric frogs; Gdovin et al., 1999, Torgerson et al., 2001, Gargaglioni and Milsom, 2007).

The aim of my work was to use the developing bullfrog as a tool to investigate the functional consequences of nicotine and ethanol exposure on the development of breathing control. An over-arching hypothesis of my work was that nicotine and ethanol impair breathing responses to

extremes in systemic gas conditions (low oxygen, O_2, and high carbon dioxide, CO_2), which was based on previous findings (Taylor et al., 2008) that nicotine and ethanol impair the centrally-mediated and whole-animal breathing responses to high CO_2. My intent was to establish causative links between nicotine- or ethanol-exposure and impaired breathing control and to establish research models that could be used to investigate the underlying mechanisms of those causations. Identifying causes of impairment during development and creating research models to compare impaired and normal neural development within the breathing control network will lead to a better understanding of the pathogenesis of SIDS, as well as the development and plasticity of breathing control.

1.1 Sudden Infant Death Syndrome: An impairment in homeostasis

SIDS is a diagnosis of exclusion. Cause of death is recorded as SIDS if an infant under 1 year of age dies and all other causes of death are ruled out (Willinger et al., 1991). It might seem counter-intuitive to investigate the "cause" of SIDS. Once a new cause of infant mortality is identified it no longer, by definition, could be a SIDS death. To eliminate SIDS entirely infant deaths must be ascribed a specific cause, which ideally

will lead to preventative interventions, because fewer unexplained deaths will result in fewer cases of SIDS.

All deaths may, to some extent, be considered the result of failed homeostasis. The lack of identifiable evidence about the specific events that result in a SIDS death suggest that SIDS infants may have difficulty simply maintaining physiological homeostasis (Harper et al., 2000, Kinney, 2009). Physiological stressors that can be accommodated in normal infants become life-threatening in a SIDS infant (Filiano and Kinney, 1994). When a physiological stressor causes a deviation from homeostasis, an organism can make a compensatory response, one that returns the system to a set-point, or an adaptive response, one that allows the system to accommodate a new set-point. Breathing responses to shifts in systemic gas conditions (either high CO_2, hypercapnia, or low O_2, hypoxia) are attenuated during sleep and prone-sleeping can decrease O_2 uptake and increase systemic CO_2 levels (Kuwaki et al., 2008, Rao et al., 2009, Kuwaki et al., 2010). SIDS deaths often occur while an infant is asleep (Hunt, 1989, Harper et al., 2000, Hauck et al., 2003, Kahn et al., 2003), and prone sleeping infants have a three to five times greater risk for SIDS (Willinger et al., 1991, Duncan et al., 2009). These observations suggest that both an infant's state of wakefulness

and position may be relevant to SIDS. Physiological stressors such as low O_2 and high CO_2 can easily occur in a sleeping infant and, if the infant is unable to generate the appropriate adaptive or compensatory response, a SIDS death may result. Thus, the pathology of SIDS may lie with impairments of homeostatic processes.

Hannah Kinney and colleagues have investigated neural morphology in SIDS infants (Kinney et al., 1983, Filiano and Kinney, 1994, Kinney et al., 2001, Paterson et al., 2006). They have identified abnormalities in the serotonergic system in the medullary raphe of SIDS infants, including a reduction in the binding of serotonin type 1_A receptors (5-HT_{1A}; Kinney et al., 2001, Kinney et al., 2005, Paterson et al., 2006). The medullary serotonergic network is involved in cardiorespiratory activity and arousal (Halmagyi and Colebatch, 1961, Doherty et al., 1996, Leonard, 1996, Jordan, 2005, Dutschmann et al., 2009, Murillo-Rodriguez et al., 2009, Hodges and Richerson, 2010). Impairments in cardiorespiratory function that contribute to the maintenance of systemic O_2 and CO_2 homeostasis, and are exacerbated by sleep state and position, may contribute to SIDS. Medullary 5-HT_{1A} receptors may play a role in central hypercapnic-sensitivity and compensatory responses to CO_2 (Severson et al., 2003, Corcoran et al., 2009). Similarly central

hypoxic responses rely on serotonergic mechanisms (Herman et al., 2000, Simakajornboon and Kuptanon, 2005, Gargaglioni et al., 2006, Dergacheva et al., 2009). To date the impaired cardiorespiratory control hypothesis is considered one of the most compelling explanations for SIDS (Hunt, 1992, Duncan et al., 2010).

Centrally, hypercapnia and hypoxia result in changes in the neural activity that signals for a breath to occur. This signal, neuroventilation, dictates the frequency, type, and pattern of breaths that ventilate a gas exchange surface. The central, or neuroventilatory, response to hypercapnia is increased breath frequency in an attempt to off load CO_2 (Nattie, 1999, Milsom, 2002, Putnam et al., 2005), and is a classic homeostatic compensatory mechanism (Corcoran et al., 2009). The central, or neuroventilatory, response to hypoxia, on the other hand, is an adaptive response where breathing is actively depressed to complement reduced metabolic demand for O_2, which is limited (Neubauer et al., 1990, Bisgard and Neubauer, 1995). The central responses to hypercapnia and hypoxia are, thus, opposite with respect to their effect on the frequency of breathing-related neural activity.

An infant with an underlying vulnerability (e.g., an impaired medullary serotonergic system) that is exposed to hypercapnia or hypoxia at a point when responses to these stressors are blunted (e.g., during sleep) may not respond adequately and death may result. The triple risk hypothesis for SIDS proposes that SIDS results when an infant with an underlying vulnerability (impaired medullary serotonergic system) is unable to respond to an exogenous stressor (hypercapnia or hypoxia) during a discrete critical period of development (Filiano and Kinney, 1994, Caddell, 2001, Kinney et al., 2001, Kinney, 2009). The development-period component of the triple risk hypothesis for SIDS may explain the prevalence of SIDS deaths in the 1st year of infant life and suggests that vulnerability, exposure, and/or response to the stressor are developmentally limited.

The triple risk hypothesis is an over arching theme in this dissertation. I sought to identify developmental changes in central responses to hypercapnia and hypoxia, to elicit vulnerabilities that resulted in impaired responses to those stressors, and to determine whether those vulnerabilities were permanent or could be resolved over time. Ultimately, I wanted to determine if nicotine or ethanol exposure could induce a temporary impairment of central responses to O_2 and CO_2

stressors in a model vertebrate breathing control network, the isolated bullfrog brainstem.

1.2 Nicotine and ethanol are neuroteratogens

Use of nicotine or ethanol during pregnancy has numerous deleterious effects on a developing fetus (West and Goodlett, 1990, Haustein, 1999, Muneoka et al., 2001, Olney et al., 2002, Pauly et al., 2004, Dwyer et al., 2008). Growth retardation, miscarriages, premature birth, and impaired neurodevelopment are just a few examples of why nicotine and ethanol are widely accepted as teratogens (West and Goodlett, 1990, Brien and Smith, 1991, Ernst et al., 2001, Ginzel et al., 2007, Henderson et al., 2007). Both nicotine and ethanol exposures also have widespread effects on all aspects of neurogenesis and neural communication (Powrozek et al., 2004, Slotkin, 2004, Shingo and Kito, 2005, Slikker et al., 2005, Aloe, 2006, Heath and Picciotto, 2009, Abreu-Villaca et al., 2010, Mooney and Miller, 2010), but their effects are distinct. These two neuroteratogens target different aspects of the central nervous system, the functional result of their exposure is dissimilar, and the mechanisms by which they alter fetal development vary.

Nicotine is an agonist of nicotinic acetylcholine receptors (nAchRs), heteropentameric ligand-gated ion channels assembled by the combination of α(α1-10), β(β1-4), γ, δ, and ε subunits (Lena et al., 1999, Shao and Feldman, 2002). nAchRs are widespread across the central nervous system (Kinney et al., 1993, Slotkin, 2004) and play a critical role in development as early as gastrulation (Lauder and Schambra, 1999). The cholinergic system helps facilitate the transition in neuronal development from primary replication to terminal differentiation (Slotkin, 1998, 1999, Slotkin et al., 2007a), and it modulates synaptogenesis and axonogenesis (Slotkin, 2004, Slikker et al., 2005). The cholinergic system is also important in maintaining the integrity and assembly of brain regions that regulate learning, memory, and behavior (Hohmann et al., 1988, Hohmann et al., 1998). Chronic stimulation of nAchRs by nicotine can alter any of these processes or result in desensitization, which attenuates normal cholinergic activity (Gentry and Lukas, 2002, Quick and Lester, 2002). Nicotine exposure also alters serotonergic neuron density and receptor subtype expression (Mihailescu et al., 2002, Say et al., 2007, Slotkin et al., 2007b, Duncan et al., 2009). Thus, although nicotine is an agonist of one specific receptor type, it can influence other neural signaling systems.

Ethanol has widespread direct and indirect effects on cell replication, neuroendocrine activity, ligand-gated ion channel signaling, neuronal migration, and neuron-glia communication (Gressens et al., 1992, Zhang et al., 2005, Camarillo and Miranda, 2008, Lee et al., 2008, Weinberg et al., 2008, Crews and Nixon, 2009, Gonzalez and Salido, 2009). At the synapse level, ethanol mimics γ-aminobutyric acid (GABA) and is an allosteric potentiator of $GABA_A$ receptors via the imidazobenzodiazepine binding site, an agonist producing widespread neural inhibition (Aguayo et al., 2002, Criswell and Breese, 2005, Breese et al., 2006, Wallner and Olsen, 2008). Ethanol also reduces activity at N-methyl-D-aspartic acid (NMDA) type glutamate receptors, which further reduces neural activity (Hoffman et al., 1990, Wirkner et al., 1999, Ron, 2004). The results of chronic exposure to ethanol are generally associated with its direct inhibitory actions (Harris and Mihic, 2004); however, chronic ethanol exposure can cause either sensitization or desensitization to multiple neurotransmitter systems (Trujillo and Akil, 1995, Faingold et al., 1998, Dubois et al., 2006, Dopico and Lovinger, 2009).

1.3 Nicotine and ethanol exposure affects breathing

The activation of nAchRs is fundamental to respiratory rhythm generation (Shao and Feldman, 2001, Shao et al., 2008). nAchRs are widespread in regions associated with breathing control (Wada et al., 1989, Kinney et al., 1993, Dominguez del Toro et al., 1994). The neuroventilatory effect of nicotine exposure in the brain varies based on the duration, developmental timing, and concentration of exposure, as well as the research preparation used to test the effect (Fernandez et al., 2002, Hafstrom et al., 2005, Brundage and Taylor, 2009). Persistent exposure to nicotine during early development impairs both central and whole-animal ventilatory responses to hypercapnia and hypoxia (Milerad et al., 1995, Hafstrom et al., 2002, Neff et al., 2004, Simakajornboon et al., 2004, Eugenin et al., 2008), and these response impairments are transient, with full recovery in rodent models by postnatal day eight (Simakajornboon et al., 2004, Eugenin et al., 2008).

The effects of ethanol on breathing have not been as extensively investigated as those of nicotine. Acutely, ethanol suppresses fetal breathing movements (Brien and Smith, 1991), and chronic prenatal ethanol exposure depresses respiratory responses to hypoxia (Dubois et al., 2006, Dubois et al., 2008a, Dubois et al., 2008b). Central responses

to hypoxia have not been specifically investigated following prenatal ethanol exposure nor have hypercapnic responses in general.

GABAergic signaling, the major target of ethanol, is fundamental to respiratory rhythm generation (Bonham, 1995, Brockhaus and Ballanyi, 1998, Ren and Greer, 2006) and may play a key role in central responses to hypercapnia and hypoxia (Richter et al., 1999, Curran et al., 2001, Li et al., 2006, Fournier et al., 2007). Both ethanol and GABA depress normal breathing activity (Gibson and Berger, 2000, Dubois et al., 2006). Acute ethanol exposure can result in a near cessation of breathing movements in the human fetus (McLeod et al., 1983, Brien and Smith, 1991). Responses to hypercapnia are thought to be produced in part through an inhibition of GABA activity (Peano et al., 1992, Horn and Waldrop, 1994, Gourine and Spyer, 2001, Liu et al., 2003, Kuribayashi et al., 2008). GABA's role in hypoxia, however, may be through the inhibition of adrenergic pathways, generating the hypoxia-induced central adaptive reduction in breathing (Fournier et al., 2007).

1.4 Animal models for neurotoxicology and breathing control

Studies investigating the effects of neuroteratogen exposures in mammals are hindered because fetal development occurs within a maternal organism. Controlling fetal exposure levels requires accounting for maternal metabolism. Rats, for example, metabolize nicotine at a faster rate than humans (Hafstrom et al., 2005). Investigators looking at prenatal exposure in rat models must use higher maternal doses to approximate comparable fetal human exposure levels (Hafstrom et al., 2005). Elevated maternal exposure may have other effects on the mother that impact the developing fetus. Monitoring fetal exposure levels can involve invasive sampling techniques that may otherwise compromise the fetus. Thus, the internal development of mammals creates several impediments to studying the developmental neurotoxicology of nicotine or ethanol with respect to breathing or a physiological process. The developing bullfrog tadpole has been used as an alternative model to evaluate the effects of developmental nicotine and alcohol exposure, because exposure conditions can be controlled for without maternal influence (Taylor et al., 2008, Brundage and Taylor, 2009).

The bullfrog tadpole develops in an aquatic environment to which teratogens like nicotine and ethanol can be added directly, and they will freely cross amphibian skin (Yorio and Bentley, 1976, Horimoto and Koyama, 1982). The concentration of nicotine or ethanol can be monitored and manipulated by sampling the water housing each tadpole (Taylor et al., 2008). Exposure can be maintained without maternal interactions for any period of time, at any point in development. This allows the testing of the direct effects of nicotine or ethanol exposure without any nonspecific effects generated by the effect of these teratogens on the mother. Furthermore, the bullfrog tadpole animal model capitalizes on the advantages of free-living developmental stages, without sacrificing physiological comparability. The cholinergic and GABAergic systems of bullfrogs are functionally similar to mammals at the receptor level and the breathing control network (Dickinson et al., 1988, Shen et al., 1994, Broch et al., 2002, Hollis and Boyd, 2003, Fournier et al., 2007, Brundage et al., 2010a, Brundage et al., 2010b), suggesting that the direct effects of nicotine or ethanol exposure at both these levels would be comparable between bullfrogs and mammals. Thus, in my studies, I opted for the bullfrog model to gain advantages of free-living development without losing the ability to extrapolate my findings to mammals, including humans.

The bullfrog has long been a research tool for physiologists. With respect to breathing, the mechanics of bullfrog ventilation are well understood as are the neural signals that stimulate each breath. Ventilatory-related motor output from the isolated bullfrog brainstem was first identified by Robert Schmidt (Schmidt, 1971), this preparation was developed and characterized independently in the 1990s (McLean et al., 1995, Galante et al., 1996, Liao et al., 1996). Since that time, the isolated bullfrog brainstem preparation has been used as a tool to study central mechanisms underlying vertebrate respiratory rhythmogenesis (Galante et al., 1996, Gdovin et al., 1999, Milsom et al., 1999, Straus, 2000, Torgerson et al., 2001, Harris et al., 2002, Winmill and Hedrick, 2003).

Central responses to both hypercapnia and hypoxia have been characterized throughout bullfrog development. Similar to mammals, bullfrogs increase lung breath frequency in response to hypercapnia and decrease breath activity with central hypoxia (Torgerson et al., 1997, Taylor et al., 2003a, Taylor et al., 2003b, Winmill et al., 2005, Fournier et al., 2007, Fournier and Kinkead, 2008). Bullfrogs are considerably more hypoxia tolerant than mammals, but despite the temporal difference in

bullfrog responses, the general effect of hypoxia on neuroventilation is consistent in frogs and mammals (Winmill et al., 2005).

Apart from control of breathing, the breathing control network in the bullfrog is an excellent research tool to investigate function of a central neural network. The isolated bullfrog brainstem can maintain a robust breathing rhythm *in vitro* for more than 2 d (personal observation). Chemical agents, temperature, pH, and ion concentrations can all be easily manipulated to gain a functional understanding of this central neural network. The consequences of exposure to different toxins at different concentrations, as well as the ability to compare direct *in vivo* and *in vitro* developmental exposure, make the developing bullfrog CNS an ideal model system for neurotoxicology and neuroplasticity research.

The effect of nicotine and ethanol exposure on bullfrog tadpole neuroventilation was first investigated following 8-12 wk of exposure to either 30 μg/L nicotine or 0.1 % ethanol (v/v) in early metamorphic tadpoles (Taylor et al., 2008). Responses to hypercapnia were impaired in both the intact animal and the isolated brainstem preparation. Taylor et al. (2008) were the first to demonstrate attenuated responses to hypercapnia following early developmental exposure to either nicotine or

ethanol in any animal model. Baseline ventilation was unaffected suggesting that the compensatory mechanism involved in responding to hypercapnia was impaired rather than normal ventilatory activity. Subsequent research showed that 3 wk of nicotine exposure was sufficient to attenuate the central hypercapnic response of early metamorphic tadpoles, but the responses of late metamorphic tadpoles and juvenile bullfrogs were unaffected by the shorter nicotine exposure (Brundage and Taylor, 2009, Brundage et al., 2010b). Furthermore, the central hypercapnic response was impaired in both early and late metamorphic tadpoles following 10 wk of nicotine exposure (Brundage and Taylor, 2009). Thus, the effects of chronic nicotine exposure on the central hypercapnic response are dependent upon the duration and developmental timing of exposure.

The effects of acute nicotine exposure on the isolated bullfrog brainstem are notably different than those of chronic treatment. Acute exposure to 18 μg/L nicotine did not alter early metamorphic tadpole neuroventilation. However, it did cause a reduction in normocapnic breath frequency in late metamorphic tadpoles and juvenile bullfrogs, and attenuated the central hypercapnic response of juvenile bullfrogs (Brundage et al., 2010a). We further demonstrated that higher

concentrations of nicotine attenuated the hypercapnic responses of the earlier developmental groups (50 and 100 μg/L for the late and early metamorphic tadpoles, respectively) and that these are specific effects arising through nAchR activation because they are blocked by mecamylamine (Brundage et al., 2010a) and similar to the effects of galantamine (Brundage et al., 2010b). Thus, our data suggest that the role of the cholinergic system in the breathing control network changes with development and may be directly involved in the regulation of central responses to hypercapnia.

1.5 Experimental design, scope, and direction

The primary aim of my research was to develop a model that would help advance our understanding of how nicotine or ethanol exposure during development may contribute to SIDS. My hypothesis was that nicotine or ethanol exposure both directly (outside of any maternal influences) impaired normal central homeostatic responses to the neuroventilatory stressors, hypercapnia and hypoxia. This was based on research suggesting impairments in hypercapnic and/or hypoxic responses contribute to SIDS (Hunt, 1992, Duncan et al., 2010). For my Master of Science degree, we characterized nicotine-induced impairment of the

tadpole central hypercapnic response (Brundage and Taylor, 2009). This doctoral dissertation extended in a natural direction from my Master's research.

In research for this dissertation, I first characterized ethanol-induced impairment of the tadpole central hypercapnic response. I tested acute, 3-wk, and 10-wk durations of chronic ethanol exposure during early and late periods of metamorphosis (see chapter two). This is the first study to evaluate the duration and timing of developmental ethanol exposure on responses to a ventilatory stressor. I then tested the same duration of exposure to nicotine and ethanol on central responses to hypoxia in early and late metamorphic tadpoles (see chapter three). This is the first study to examine the effects of ethanol exposure on central responses to hypoxia. I also quantified the hypoxic effect on other breathing parameters (gill breaths, durations, and amplitudes) to determine if chronic neuroteratogen exposure had any other effect on tadpole neuroventilation. In a final set of experiments, I determined if central responses to hypercapnia could return if a teratogen-free period of development was allowed to occur (see chapter four).

Future research that extends in a natural direction from this dissertation should investigate the mechanisms by which central ventilatory responses are able to recover as well as the mechanisms by which they are impaired by chronic exposure to nicotine or ethanol. Investigating mechanisms of both impairment and recovery will provide important insight into the neural network that controls breathing and the capacity for neuroplasticity within that network. The results of the studies presented here meet the aim of establishing a model that can be used to identify the mechanisms by which nicotine or ethanol impairs a centrally mediated homeostatic response and how those responses are able to recover.

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CHAPTER TWO

Chronic ethanol exposure during development impairs central hypercapnic ventilatory drive[1]

2.1 Abstract

Chronic ethanol exposure early in development is a Sudden Infant Death Syndrome (SIDS) risk factor, it is unclear how ethanol exposure may contribute to SIDS cases; however, ethanol may act to impair drives that maintain homeostasis. Central hypercapnic ventilatory drive (CHVD) potentiates ventilation to mitigate acidosis. We tested the hypothesis that chronic ethanol exposure impairs CHVD in bullfrog tadpoles. Early and late metamorphic tadpoles were exposed *in vivo* to 0.12 - 0.06 g/dL for either 3- or 10-wk durations. Brainstems from these animals were isolated at the end of exposure, and the neural correlates of ventilation were recorded *in vitro* during superfusion with normocapnic (1.5 % CO_2: 98.5 % O_2) and hypercapnic (5.0 % CO_2: 95.0 % O_2) artificial cerebral spinal fluid. Normocapnic neuroventilation was unaffected by chronic

[1]Brundage, C.M. and Taylor, B.E. 2010. Chronic ethanol exposure during development impairs central hypercapnic ventilatory drive.(formatted for submission to Neuroscience).

ethanol exposure. Central ventilatory potentiation was lost in response to hypercapnia following 10 but not 3 wk of ethanol exposure in both early and late metamorphic tadpoles. The neuroventilatory effects of chronic ethanol exposure were distinguishable from those of acute central ethanol (0.08 g/dL) exposure, which attenuated early metamorphic tadpole normocapnic neuroventilation, but had no effect on tadpole CHVD. Thus, 10 wk of ethanol exposure both early and late in metamorphosis impairs CHVD in bullfrog tadpoles.

2.2 Introduction

Breathing is a vital homeostatic process modulated primarily by the need to neutralize the acidosis created by CO_2 produced by the catabolism of metabolic fuels (Nattie, 1999, McCrimmon et al., 2000, Feldman et al., 2003). An inability to adequately compensate for a homeostatic deviation is hypothesized to be an underlying cause of Sudden Infant Death Syndrome (SIDS; Bolton, 1990, Filiano and Kinney, 1994, Jin et al., 2006). Central hypercapnic acidosis elicits an increase in the rate of ventilation to expedite exhalation of CO_2 (O'Regan and Majcherczyk, 1982, Milsom, 1995, Nattie, 1999, Putnam et al., 2004, Putnam et al., 2005). This central hypercapnic ventilatory drive (CHVD) is one of many

fundamental processes that may be inadequate in SIDS cases (Shannon et al., 1977, Dunne et al., 1992, Richerson et al., 2001, Jin et al., 2006). The underlying cause for this impairment is currently unknown, but early developmental ethanol exposure is considered a SIDS risk factor (Iyasu et al., 2002, Kinney et al., 2003).

Responsiveness to the ventilatory stressor, hypoxia, is decreased following chronic developmental ethanol exposure (Dubois et al., 2008, Kervern et al., 2008). Fewer studies have looked at the effect of ethanol on hypercapnic responses and CHVD, despite evidence that ethanol's action on γ-aminobutyric acid (GABA) neurotransmission may alter GABA's alleged role in disinhibiting responses to hypercapnia (Peano et al., 1992, Horn and Waldrop, 1994, Gourine and Spyer, 2001, Liu et al., 2003, Kuribayashi et al., 2008). Acute ethanol lowers the response threshold of CHVD in adults, but acute, late-gestational exposure has no impact on fetal hypercapnic drive (Sahn et al., 1975, Michiels et al., 1983, Marchal and Droulle, 1988). Investigating the effect of chronic ethanol exposure on responsiveness of the respiratory neural circuit to the stressor hypercapnia could provide information about the causes of SIDS.

Ethanol is a neuroteratogen that causes a wide range of adverse effects on development including central nervous system dysfunctions comprising cognitive impairments, behavioral disturbances, and neurological damage (Clarren et al., 1978). Prenatal ethanol exposure is associated with alterations in autonomic control that occur during sleep soon after birth (Fifer et al., 2009). The neuroventilatory consequences of developmental ethanol exposure depend on the timing of exposure relative to development. Acute ethanol exposure nearly abolishes fetal breathing movements in late but not early gestation (Goodlett et al., 2005). Chronic late-gestational ethanol exposure results in numerous neuronal deficits (Marchal and Droulle, 1988, Smith et al., 1990, Brien and Smith, 1991, Hamre and West, 1993, Marcussen et al., 1994, Watson et al., 1996, Goodlett and Eilers, 1997, Goodlett et al., 1998), while early gestational ethanol exposure confers the greatest risk of SIDS (Iyasu et al., 2002). Ethanol exposure during different periods of brain development results in regional differences in cell loss (West et al., 1990, Maier et al., 1999), and this could account for disparate ethanol vulnerability, based in the developmental timing and duration of exposure, of the neural network controlling breathing.

To our knowledge the only evaluation of the consequences of chronic developmental ethanol exposure on CHVD was done in early metamorphic bullfrog tadpoles (Taylor et al., 2008). Developing bullfrog tadpoles have been used as a model to evaluate the consequences of teratogen exposures (Beatty et al., 1976, Neal et al., 1979, Schuytema et al., 1991, Brundage, 2008). Tadpoles continuously exposed to 0.1 % ethanol (v/v) for 8-12 wk throughout development failed to respond normally to hypercapnic challenges *in vivo* and *in vitro* (Taylor et al., 2008). It was unclear, however, whether this response was based on the developmental stage of the tadpoles, the duration of ethanol exposure, or an acute reaction to ethanol. The present study was designed to clarify these issues. Tadpoles from both early and late periods of metamorphosis were exposed to ethanol for either 3- or 10-wk durations prior to assessing CHVD in their isolated brainstems. Responses were compared with control tadpoles and those of brainstems acutely exposed to ethanol via bath application. We hypothesized that early metamorphic tadpoles would demonstrate an increased susceptibility to chronic ethanol exposure, and that CHVD would be impaired in a fashion distinguishable from that of late metamorphic tadpoles and brainstems acutely exposed to ethanol. This hypothesis was based on the early period of developmental ethanol exposure being more closely linked with

SIDS (Iyasu et al., 2002) and early metamorphic tadpoles being more susceptible to CHVD impairments following nicotine exposure, another SIDS risk factor (Taylor et al., 2008, Brundage and Taylor, 2009).

2.3 Experimental Procedures

Animals

Studies were performed on *Lithobates* (formerly *Rana*) *catesbeiana* tadpoles (n = 108) purchased from a commercial supplier (Sullivan Co. Inc., www.researchamphibians.com). Tadpoles were maintained at room temperature and were fed goldfish food daily. Tadpoles were housed for 10 wk in aquaria with either dechlorinated water only or dechlorinated water containing ethanol (0.12 - 0.06 g/dL). The ethanol concentration varied due to the volatilization of ethanol from the tank. This range encompasses the concentration of a previous study using bullfrogs (Taylor et al., 2008) and the 0.08 g/dL blood alcohol content that is the legal definition of alcohol intoxication in many western countries (Caldeira et al., 2004). 3-wk ethanol exposure was achieved by maintaining tadpoles in dechlorinated water for 7 wk before transferring them to ethanol-containing water for 3 wk. Consequently, all animals

were maintained in the laboratory for 10 wk regardless of the duration of ethanol exposure. Taylor et al. (2008) reported impairments of hypercapnic response after 8 to 12 wk of ethanol exposure. We wanted to reproduce those findings and determine if CHVD impairment is stage- or duration-dependent; thus, the mid-range10-wk exposure was selected. 3-wk ethanol exposure was chosen to determine whether a significantly shorter exposure could lead to a similar impairment.

The developmental stage of each tadpole was determined twice, once at the start of treatment and again at the time of dissection to ensure developmental homogeneity of the treatment groups. At the time of dissection each tadpole was either "early metamorphic" (forelimbs absent, hind limbs paddle-like without joints or separated toes) or "late metamorphic" (forelimbs and hind limbs present, tail being resorbed), which corresponded to developmental stages 7 - 12 or 20 - 25, respectively, in the classification scheme of Taylor and Köllros (1946). Tadpoles included in early metamorphic groups were stages 7 - 9 at the start of their 10-wk laboratory maintenance, while those included in late metamorphic groups were stages 18 - 20. This was true for animals that received either 3 or 10 wk of chronic ethanol exposure, as they were all maintained for 10 wk. Animals that did not remain within these stage

ranges were excluded from the datasets. All animal care and experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Alaska Fairbanks and complied with all state and federal ethical guidelines

Surgical preparation

Each tadpole was anesthetized by immersion for 1-2 min in cold (4 °C) 0.2 mM tricaine methanesulfonate (MS222; Sigma, www.sigmaaldrich.com) in dechlorinated water buffered to pH 7.8 with $NaHCO_3$. The front of the head rostral to the nares and the back of the body (hind limbs and tail, if present) were removed. The dorsal cranium and forebrain rostral to the diencephalon were resected and the fourth ventricle opened by removing the choroid plexus. The remaining brainstem and spinal cord were removed *en bloc* and further trimmed rostrally to the optic tectum and caudally to the brachial nerve. During dissection, exposed tissues were superfused with cold artificial cerebral spinal fluid (aCSF) composed of (in mM) 104 NaCl, 4 KCl, 1.4 $MgCl_2$, 10 D-glucose, 25 $NaHCO_3$ and 2.4 $CaCl_2$ equilibrated with 100 % O_2. The aCSF HCO_3^- concentration is similar to that of plasma from late metamorphic tadpoles and frogs but higher than that in plasma from

early metamorphic tadpoles (Just et al., 1973). This HCO_3^- concentration has been used in previous tadpole studies (Taylor et al., 2003a, 2003b, 2008) and was selected here to ensure comparability with previous studies and between experiments on animals of different metamorphic stages.

The isolated brainstem was transferred *en bloc* to a 2.5 ml, Plexiglas, flow-through recording chamber and was supported, ventral side up, between coarse nylon mesh such that all surfaces were bathed with aCSF flowing from rostral to caudal at a rate of 5 ml/min. A supply of aCSF, equilibrated with O_2-CO_2 mixtures that produced the desired pH, flowed through plastic tubing to the recording chamber and bathed the isolated brainstem. The pH of the aCSF was maintained at either pH 7.8 (1.5 % CO_2: 98.5 % O_2; normocapnia) or pH 7.4 (5.0 % CO_2: 95.0 % O_2; hypercapnia) by adjusting the fractional concentrations of O_2 and CO_2 in the equilibration gas. CO_2 was monitored with a CO_2 analyzer (Capstar 100; CWE, www.cwe-inc.com). After isolation the brainstem was allowed to stabilize for 1 h while superfused at 23 °C, with aCSF of pH 7.8 (~9 torr P_{CO_2}).

Nerve recording

Roots of the facial and hypoglossal nerves were drawn into glass suction electrodes pulled from 1-mm-diameter capillary glass to tip diameters that fit the nerve roots. Whole-nerve discharge was amplified (X100 by DAM 50 amplifiers, World Precision Instruments, www.wpiinc.com; X1000 by a model 1700 amplifier, A-M Systems, www.a-msystems.com) and filtered (100 Hz high pass to 1 kHz low pass). The amplified and filtered nerve output was sent to a data acquisition system (Powerlab, AD Instruments, www.adinstruments.com), which sampled at 1 kHz. Data were archived as whole-nerve discharge, and duplicate integrated (full-wave rectified and averaged over 200 ms) neurograms were acquired simultaneously. Such recordings were made during the initial 1-h post-isolation stabilization period and recorded continuously throughout the duration of each treatment protocol.

Treatment groups

Chronic ethanol-exposed animals contributed to four distinct brainstem groups: early and late metamorphic 3-wk ethanol-exposed brainstems (n = 13 and n = 14, respectively), and early and late metamorphic 10-wk

ethanol-exposed brainstems (n = 13 and n = 14, respectively). About half of each chronically exposed group of brainstems received a hypercapnic challenge *in vitro* after isolation from the animal (early and late metamorphic 3-wk ethanol-exposed, n = 8 and n = 8, respectively; early and late metamorphic 10-wk ethanol-exposed, n = 7 and n = 7, respectively). Neurograms were recorded during 30 min of normocapnia, followed by 30 min of hypercapnia, followed by a 30 min return to normocapnia. In this way putative baseline ventilation, response to hypercapnia, and recovery from hypercapnia were evaluated for each brainstem in a single 90-min protocol. The remainder of each chronically exposed group of brainstems, as well as brainstems from unexposed early and late metamorphic tadpoles (n = 6 for both groups) served as time-controls. These preparations remained normocapnic throughout the experiment. In this way the robustness of neuroventilation over the 90-min period of experimentation, despite chronic ethanol exposure, was confirmed.

Unexposed animals (tadpoles held for 10 wk without exposure to ethanol) contributed to 8 brainstem groups. As described above, 2 groups served in the time-control experiment, and 2 groups served as hypercapnic treatment-controls for assessing the effects of CHVD in control animals.

Four groups of unexposed brainstems were used to assess the neuroventilatory effects of acute ethanol exposure.

Two groups of early and 2 groups of late metamorphic unexposed tadpoles contributed brainstems for assessment of acute ethanol effects on neuroventilation. One early and 1 late metamorphic group (n = 8 for both) received the hypercapnic challenge described above, were allowed to recover for 60 min under normocapnic conditions, then the aCSF superfusate was switched to one containing 0.08 g/dL for 30 min of normocapnia and a 30-min hypercapnic challenge, with final recovery occurring during superfusion with ethanol-free aCSF. Neurograms were recorded throughout this 210-min treatment protocol, which allowed us to compare CHVD before and after acute ethanol exposure. It has previously been shown that early and late metamorphic tadpoles respond to sequential hypercapnic challenges with statistically significant and indistinguishable increases in lung burst frequency (Brundage et al., 2010). To confirm that finding and provide a control for the dual hypercapnic challenge, 1 early and 1 late metamorphic group (n = 6 for both) underwent the same dual hypercapnic challenge but received only ethanol-free aCSF.

Data and statistical analyses

Activity bursts in the neurograms were designated as either putative gill or putative lung breaths on the basis of the amplitude of the integrated nerve activity and the presence or absence of coincident firing in both the facial and hypoglossal nerves, as previously described (Torgerson et al., 1998, Taylor et al., 2003a, 2003b). Putative gill breaths had lower integrated burst amplitude on the facial nerve and little or no coincident burst activity in the hypoglossal nerve. Putative lung breaths had higher integrated burst amplitude in the facial nerve and coincident burst activity in the hypoglossal nerve. Taylor et al. (2003a, 2003b) demonstrated that increased frequency of putative lung breaths is the primary manifestation of the CHVD; the duration and amplitude of putative lung breaths and the frequency, duration, and amplitude of putative gill breaths are unaffected by hypercapnia. Thus, we quantified only the frequency of lung bursts by calculating the mean number of lung bursts per minute over the final 3 minutes of a 30-min brainstem treatment of CO_2/pH (normocapnia or hypercapnia) and aCSF (ethanol or ethanol-free).

Frequencies of lung bursts were compared using repeated-measures analysis of variance (RM-ANOVA; SigmaStat, www.systat.com). When RM-ANOVA indicated that significant differences existed between the chronic treatment conditions, multiple comparisons were made using the Holm-Sidak multiple comparison test. Frequencies of lung bursts in acute ethanol-exposed brainstems were also analyzed using a 2-way RM-ANOVA to determine if the presence of ethanol affected responses to CO_2. Relative changes in lung burst frequency in response to hypercapnia were determined by expressing the change in lung burst frequency between normocapnia to hypercapnia by the mean normocapnic lung burst frequency for each group. Comparisons between early and late metamorphic tadpoles and between chronic ethanol-exposed and unexposed groups were conducted using t-test comparisons (SigmaStat, www.systat.com). Values reported in the text are always means ± SE.

2.4 Results

Normocapnic lung burst frequency was unaffected by chronic ethanol exposure

Chronic ethanol exposure did not affect normocapnic lung burst frequency in early metamorphic brainstems. Lung burst frequency in early metamorphic brainstems, both those that had been previously exposed to 0.12 - 0.06 g/dL ethanol for 3 or 10 wk and those unexposed, was monitored for 90 min under normocapnic conditions (Fig. 2.1). Normocapnic lung neuroventilatory frequency in unexposed brainstems (0.68 ± 0.28 bursts/min) was not significantly different from that of early metamorphic brainstems exposed to ethanol for 3 wk (1.3 ± 0.9 bursts/min; $p = 0.24$) or 10 wk (1.1 ± 1.7 bursts/min; $p = 0.55$). The lung burst frequency of time-control experiments from all 3 early metamorphic groups did not vary significantly over the 90-min period of experimentation ($p = 0.61$, $p = 1.00$, and $p = 0.39$ for control, 3-wk, and 10-wk ethanol-exposed brainstems, respectively). Lung neuroventilation in early metamorphic tadpole brainstems was consistently robust for over 90 min regardless of whether or not the animal was chronically exposed to ethanol for 3 or 10 wk.

Chronic ethanol exposure did not affect the normocapnic lung burst frequency in late metamorphic brainstems (Fig. 2.1). Lung burst frequency in late metamorphic brainstems was significantly greater than that in early metamorphic brainstems (7.6 ± 3.0 bursts/min; $p < 0.01$). The lung burst frequency in unexposed late metamorphic brainstems and those exposed to ethanol for 3 wk was not significantly different (7.6 ± 3.2 bursts/min; $p = 1.00$). There was no significant change in the lung burst frequency in late metamorphic brainstems over the 90-min period of experimentation ($p = 0.67$, $p = 0.06$, and $p = 0.10$ for control, 3-wk, and 10-wk ethanol-exposed brainstems, respectively). Similar to early development, lung neuroventilation in late metamorphic brainstems is consistently robust for over 90 min regardless of whether or not the intact animal was chronically exposed to ethanol for 3 or 10 wk.

10-wk ethanol exposure impaired central hypercapnic ventilatory drive

Ten-wk, but not 3-wk, ethanol exposure blocked the hypercapnic response of early metamorphic brainstems. Unexposed brainstems exhibited significantly increased lung burst frequency in response to

hypercapnia (Fig. 2.2A; from 0.5 ± 0.10 to 1.5 ± 0.4 bursts/min; $p < 0.01$). Brainstems from early metamorphic tadpoles continuously exposed to 0.12 - 0.06 g/dL ethanol for 3 wk responded similarly by significantly increasing lung burst frequency in response to hypercapnia (from 0.8 ± 0.4 to 1.5 ± 0.6 bursts/min; $p = 0.02$). Early metamorphic brainstems that had been exposed to ethanol for 10 wk failed to respond to hypercapnia (0. 7 ± 0.2 bursts/min during normocapnia and 0.7 ± 0.3 bursts/min during hypercapnia; $p = 0.82$). 10-wk ethanol exposure impaired CHVD in early metamorphic tadpoles.

Ten-wk, but not 3-wk, chronic ethanol exposure blocked the hypercapnic response of late metamorphic brainstems. Unexposed late metamorphic brainstems increased the frequency of lung bursts in response to hypercapnia (Fig. 2.2B; from 8.4 ± 1.3 to 15.9 ± 1.8 bursts/min; $p < 0.001$). Three-wk ethanol exposure did not block this hypercapnic response in late metamorphic brainstems, which increased from 4.8 ± 0.9 to 8.8 ± 1.1 bursts/min ($p < 0.01$). Late metamorphic brainstems exposed to ethanol for 10 wk failed to increase lung burst frequency in response to hypercapnia (3.6 ± 1.8 bursts/min during normocapnia and 3.4 ± 1.7 bursts/min during hypercapnia; $p = 0.96$). Thus, CHVD

impairment by 10-wk but not 3-wk ethanol exposure was consistent across tadpole metamorphosis.

Acute ethanol decreased normocapnic lung burst frequency but not central hypercapnic ventilatory drive

Impact of acute ethanol treatment on the frequency of lung neuroventilation was used to compare chronic ethanol CHVD impairments with the acute effect of ethanol. Lung burst frequency was quantified for each minute in the 210-min acute ethanol-exposure protocol (Fig. 2.3). Acute ethanol significantly decreased normocapnic lung burst frequency in early metamorphic brainstems (Fig. 2.4A; from 0.8 ± 0.2 to 0.4 ± 0.2 bursts/min; $p < 0.01$), but did not alter normocapnic lung burst frequency in late metamorphic brainstems (Fig. 2.4B; 7.0 ± 2.0 bursts/min prior to ethanol and 5.4 ± 1.6 bursts/min during ethanol exposure; $p = 0.38$). Thus, acute ethanol exposure had a development-dependent effect on normocapnic lung neuroventilation; decreased lung burst frequency was limited to early development.

Unlike 10-wk chronic ethanol treatment, acute ethanol exposure had no effect on the hypercapnia-induced increase in lung burst frequency.

Acutely exposed early metamorphic brainstems demonstrated a significant increase in lung burst frequency in response to hypercapnia (Fig. 2.4A; 2.0 ± 0.9 bursts/min; $p < 0.01$). The presence or absence of ethanol in the aCSF had no significant effect on the CHVD in these brainstems (1.5 ± 0.6 bursts/min before ethanol and 2.0 ± 0.9 bursts/min during ethanol; $p = 0.18$). Unexposed early metamorphic brainstems that received the dual protocol without acute ethanol exposure responded to and recovered from both hypercapnia challenges; they exhibited a similar significant increase in lung burst frequency (1.9 ± 0.9 bursts/min during the first hypercapnic challenge and 1.9 ± 0.6 bursts/min during the second hypercapnic challenge; $p = 0.86$). The first hypercapnic challenge did not sensitize or acclimatize the brainstem to high CO_2, either could have masked an effect of ethanol on CHVD. Therefore, acute exposure to 0.08 g/dL ethanol depressed normocapnic lung neuroventilation without significantly affecting CHVD in early metamorphic brainstems.

Late metamorphic brainstems also exhibited a statistically similar and significant CHVD before and during acute ethanol exposure, and lung burst frequency was significantly increased by hypercapnia (Fig. 2.4B; 18.6 ± 3.3 bursts/min during a first hypercapnic challenge without

ethanol and 16.2 ± 2.5 bursts/min during a second hypercapnic challenge with ethanol; p = 0.58). There was not a significant interaction between acute ethanol exposure and the level of CO_2 in the aCSF (p = 0.89). Thus, similar to early metamorphosis, acute exposure to 0.08 g/dL ethanol had no significant effect on CHVD in late metamorphic brainstems.

Central hypercapnic ventilatory drive was consistent throughout development despite acute and 3-wk ethanol exposure

Thus far we have compared lung burst frequency only among developmentally matched brainstems. A steady increase in lung burst frequency across bullfrog tadpole development has been demonstrated (Taylor et al., 2003b). Therefore, to compare CHVD at different stages of development, we calculated relative hypercapnic responses by expressing the hypercapnia-induced increase in lung burst frequency as a percentage of lung burst frequency during normocapnia. To minimize the effect of the very low normocapnic lung burst frequency exhibited by a few animals, the difference in each animal's lung burst frequency during normocapnia and hypercapnia was divided by the mean normocapnic lung burst frequency for the treatment group. This did not affect the

results of t-tests comparing the groups and provided a more representative depiction of CHVD in each group.

Unexposed early metamorphic brainstems increased lung burst frequency 176 ± 62 % in response to hypercapnia. Similarly, both acute and 3-wk ethanol-exposed early metamorphic brainstems exhibited increases during hypercapnia that were statistically similar to those of controls (Fig 2.5; 450 ± 200 %; $p = 0.09$ and 86 ± 30 %; $p = 0.09$, respectively). 10-wk ethanol-exposed early metamorphic brainstems exhibited no significant CHVD (lung burst frequency during hypercapnia was 11 ± 46 % greater than during normocapnia), and this was significantly lower than the hypercapnia-induced frequency increases exhibited by the other early metamorphic brainstems ($p = 0.09$).

CHVD in late metamorphic brainstems was also unaffected by acute and 3-wk ethanol exposure. Lung burst frequency in unexposed as well as acute and 3-wk ethanol-exposed late metamorphic brainstems increased 90 ± 12 %, 98 ± 29 %, and 81 ± 17 %, respectively (Fig. 2.5), and all preparations increased lung burst frequency in response to hypercapnia. The hypercapnia-induced increases in acute and 3-wk ethanol-exposed brainstems were not statistically different from that of the unexposed

brainstems ($p > .05$ for both). Among the 10-wk ethanol-exposed late metamorphic brainstems, 5 of 7 failed to increase lung burst frequency during hypercapnia, and the group's mean change during hypercapnia was -4 ± 70 %. Thus, the hypercapnic effect on lung burst frequency exhibited by 10-wk ethanol-exposed late metamorphic brainstems was significantly different from that of the other late metamorphic groups ($p = 0.02$).

When early and late metamorphic brainstems were compared, there were no significant differences in CHVDs in brainstems that received the same ethanol exposure ($p = 0.18$, $p = 0.10$, $p = 0.89$, $p = 0.85$ for unexposed, acute, 3-wk, and 10-wk ethanol-exposed brainstems, respectively). Thus, CHVD was consistent from early to late metamorphosis; it consistently elicited a 147 ± 58 % increase in lung burst frequency. This consistency was not affected by ethanol exposure. Extending this statement to 10-wk ethanol-exposure, which negated CHVD in early and late metamorphic brainstems, may be somewhat pedantic. Nonetheless, these data show that the central hypercapnic response did not change with tadpole development and was impaired by ethanol only when exposure was chronic for 10 wk.

2.5 Discussion

Effects of chronic ethanol exposure on central hypercapnic ventilatory drive

Ten-wk, but not 3-wk, exposure to 0.12 - 0.06 g/dL ethanol during development impaired the central hypercapnic ventilatory drive (CHVD) of both early and late metamorphic tadpoles without affecting normocapnic lung burst frequency. The neuroventilatory impairment was distinguishable from acute ethanol exposure, which decreased the normocapnic lung burst frequency of only early metamorphic tadpoles without altering CHVD. Our findings suggest that the duration of ethanol exposure during development is a critical factor in determining the ventilatory consequences of developmental ethanol exposure. The ethanol-induced CHVD impairments in early and late metamorphic tadpoles were not significantly different. Both early and late metamorphic tadpoles significantly increased their lung burst frequency in response to hypercapnia following 3-wk ethanol exposure but failed to respond significantly to hypercapnia following 10-wk ethanol exposure.

Three-wk chronic ethanol exposure was not sufficient to impair CHVD; the minimum duration of ethanol exposure necessary to affect hypercapnic ventilation is unknown, as is the minimum concentration of ethanol needed to impair CHVD. In mammalian studies, chronic ethanol exposure fluctuates with maternal consumption as well as maternal metabolism and cardiovascular perfusion. In this study the level of ethanol exposure was kept relatively constant. One of the benefits of this animal model is the ability to control and manipulate the level of ethanol exposure. An important future direction is to determine the minimum exposure needed to induce CHVD impairments and whether intermittent exposure to ethanol over the 10-wk exposure duration would produce a similar neuroventilatory impairment.

Our finding and those of Taylor et al. (2008) that chronic ethanol exposure impairs the tadpole hypercapnia response represent the only known studies on the effects of developmental ethanol exposure on central breathing control responses to CO_2. In rats, chronic prenatal ethanol exposure decreased minute lung ventilation due to a reduction in expiratory duration (Dubois et al., 2006). Normocapnic ventilation was unaffected following chronic ethanol exposure in the tadpole. Differences in baseline ventilation between these studies may be attributed to

species differences, or variations in the design and scope of each experiment. It is interesting to note that there is no published report of minute ventilation being altered in infants diagnosed with fetal alcohol syndrome.

Effects of acute ethanol exposure on central hypercapnic ventilatory drive

Acute ethanol treatment was used to distinguish the effects of chronic ethanol exposure from the physiological response to acute ethanol. Acute exposure significantly depressed the normocapnic lung burst frequency of brainstem preparations from early but not late metamorphic tadpoles so severely that lung ventilation was nearly abolished. Maternal ethanol consumption during the third trimester of gestation elicits a similar reaction with an inhibition of fetal breathing movements (Marchal and Droulle, 1988, Smith et al., 1990, Brien and Smith, 1991, Watson et al., 1996). The respiratory output of the neonatal rat brainstem slice preparation is also inhibited by acute ethanol exposure primarily via the action of ethanol on respiratory-related glycine, GABA, and NMDA receptor activity (Gibson and Berger, 2000). The acute ethanol-induced decrease in lung burst frequency of early metamorphic tadpoles was fully

reversed within the 30-min washout period, which confirms that bath application of normal (i.e., ethanol-free) aCSF during the stabilization period after brainstem isolation in chronic ethanol-exposed tadpoles groups was sufficient to wash out any extracellular ethanol. This assures that the chronic effects we report are due to a neurophysiological change induced by ethanol rather than an *in situ* effect of this chemical. There may also be some sensitization to acute ethanol in the long-term ethanol exposed preparations. Chronic early developmental ethanol exposure has been found to alter the sensitivity of the rat respiratory network to acute ethanol (Dubois et al., 2006).

Acute ethanol exposure, like 3-wk chronic exposure, had no effect on CHVD; early and late metamorphic tadpoles significantly increased their lung burst frequency in response to hypercapnia during acute ethanol exposure. The relative CHVD of acute ethanol-exposed early metamorphic tadpoles was considerable (450 ± 200 %). This increase may be due to a central drive to overcome the normocapnic ventilatory depression induced by acute ethanol exposure. Such a compensation would suggest that early metamorphic tadpoles may increase lung ventilation to a threshold level in response to hypercapnia in spite of an ethanol-evoked ventilatory depression. Alternatively the CHVD response

mechanism of early metamorphic tadpoles may be selectively upregulated by acute ethanol.

The CHVD of bullfrog tadpoles was consistent across development; hypercapnia induced a 2- to 3-fold increase in lung burst frequency in brainstems isolated from early and late metamorphic tadpoles. Furthermore, this consistency persisted despite acute or 3-wk ethanol exposure. In several species, CO_2 sensitivity increases with development but this change is not consistent: CO_2 sensitivity is low in newborn mice and dogs, then increases to adult levels in the postnatal period; it is high in newborn rats and pigs, drops over the first 1-2 wk of development, and then increases to adult levels in the postnatal period; it is consistent across development in humans; and it hasn't been investigated across development in most species (for review see Putnam et al., 2005).

In rats, investigation of CO_2 sensitivity has been conducted at the cellular level, and again the results are confounding. The abundance of CO_2-sensitive neurons and the sensitivity of each neuron seem to be stable during development in some chemosensitive sites (Stunden et al., 2001); however, there is a developmental increase in the abundance of CO_2-sensitive neurons in the parvocellular lateral reticular nucleus

(Wickstrom et al., 2002) and in the CO_2 sensitivity of raphe neurons (Wang and Richerson, 1999). Variability in the developmental stability of CO_2 sensitivity and the mechanisms of its change may reflect a scope for neuroplasticity in the respiratory CO_2 response, one that could compensate for a deficiency in the respiratory neural control of CO_2/pH homeostasis. Whether early or late metamorphic tadpoles have sufficient neuroplasticity to recover CHVD following ethanol exposure or if the impaired hypercapnic response persists throughout development remains to be determined.

Amphibians are unique in that their free-living development includes a transition from O_2-driven aquatic respiration to CO_2-driven aerial respiration. The consistency of bullfrog CO_2 sensitivity across ontogeny suggests responses to CO_2 may develop concurrently with the onset of respiratory rhythm generation. Although the net effect of ethanol exposure on CHVD did not differ between early and late metamorphic tadpoles in this study, the consequences of that impairment may have been greater in the more aerial-dependent (CO_2-driven) late metamorphic tadpoles.

Chronic ethanol is a ventilatory neuroteratogen

Ethanol does not target a specific receptor type; there is considerable evidence that chronic ethanol directly or indirectly alters a number of neurotransmitter pathways most notably affecting glutamatergic, glycinergic, catecholaminergic, and GABAergic transmission (Suzdak et al., 1986, Hoffman et al., 1989, Lovinger et al., 1989, Hoffman et al., 1990, Eckardt et al., 1998, Ikonomidou et al., 1999, Harris and Mihic, 2004, Badanich et al., 2007, Lobo and Harris, 2008). Chronic ethanol exposure may alter the function of any or all of these systems in the developing tadpoles. The mechanism(s) by which ethanol impairs CHVD is the focus of other investigations in our laboratory, with an emphasis on $GABA_A$ receptors because chronic developmental ethanol exposure potentiates $GABA_A$ receptors. (Ikonomidou et al., 2000, Olney et al., 2002, Sanderson et al., 2009). Both this GABAergic potentiation as well as an ethanol-induced inhibition of NMDA receptor activation may, with time, result in a general neuronal inhibition altering hypercapnic response mechanisms. GABAergic inhibition has been linked with CHVD; the potentiation of $GABA_A$ receptors may attenuate the inhibition of GABAergic transmission necessary to adequately respond to hypercapnia (Peano et al., 1992, Gourine and Spyer, 2001, Liu et al., 2003,

Kuribayashi et al., 2008). It remains to be seen whether GABAergic transmission is altered in tadpoles following chronic ethanol exposure.

The observed deficiencies in CHVD could result from impaired CO_2 chemoreception and/or impaired compensatory response mechanisms. Chronic ethanol exposure does not lower normocapnic ventilation, which itself is driven by normocapnic levels of metabolically produced CO_2, suggesting that sensing CO_2 is not significantly impaired and normal ventilatory mechanisms are intact. Consequently, chronic ethanol exposure may affect compensatory response mechanisms, specifically the drive to increase lung burst frequency in response to hypercapnia. Similar impairments in compensatory response mechanisms are hypothesized to contribute to SIDS (Bolton, 1990, Filiano and Kinney, 1994, Jin et al., 2006), and developmental ethanol exposure is a SIDS risk factor (Iyasu et al., 2002, Kinney et al., 2003). Gestational ethanol exposure may act to impair similar compensatory response mechanisms in humans, thereby limiting the ability of infants to adequately respond to hypercapnia. Early developmental ethanol exposure depresses rat hypoxic ventilatory compensatory responses (Dubois et al., 2008). It will be interesting to see if developmental ethanol exposure impairs the hypoxic response in tadpoles.

2.6 Conclusions

Chronic developmental ethanol exposure impairs the central ventilatory CO_2 response of early and late metamorphic bullfrog tadpoles following 10 but not 3 wk of continuous exposure. Neither the frequency nor the consistency of normocapnic ventilation is affected by chronic ethanol exposure. The ventilatory consequences of chronic ethanol treatment differ from that of acute treatment, which decreases the lung burst frequency of only early metamorphic tadpoles without affecting responses to hypercapnia. The specific action of ethanol and the central hypercapnic ventilatory response are only partially understood; nonetheless this study offers insight into developmental ethanol-associated pathologies and illustrates the teratogenic effects of chronic ethanol exposure on a ventilatory compensatory response mechanism.

2.7 Acknowledgements

Dr. Barbara E. Taylor served as my advisor and was the principal investigator for the laboratory overseeing this research. The authors would like to thank Mr. Justin C. Buehner for his help generating data

used in this study. This work was supported by NIH-NINDS grant # U54 NS041069-06A1.

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2.9 Figures

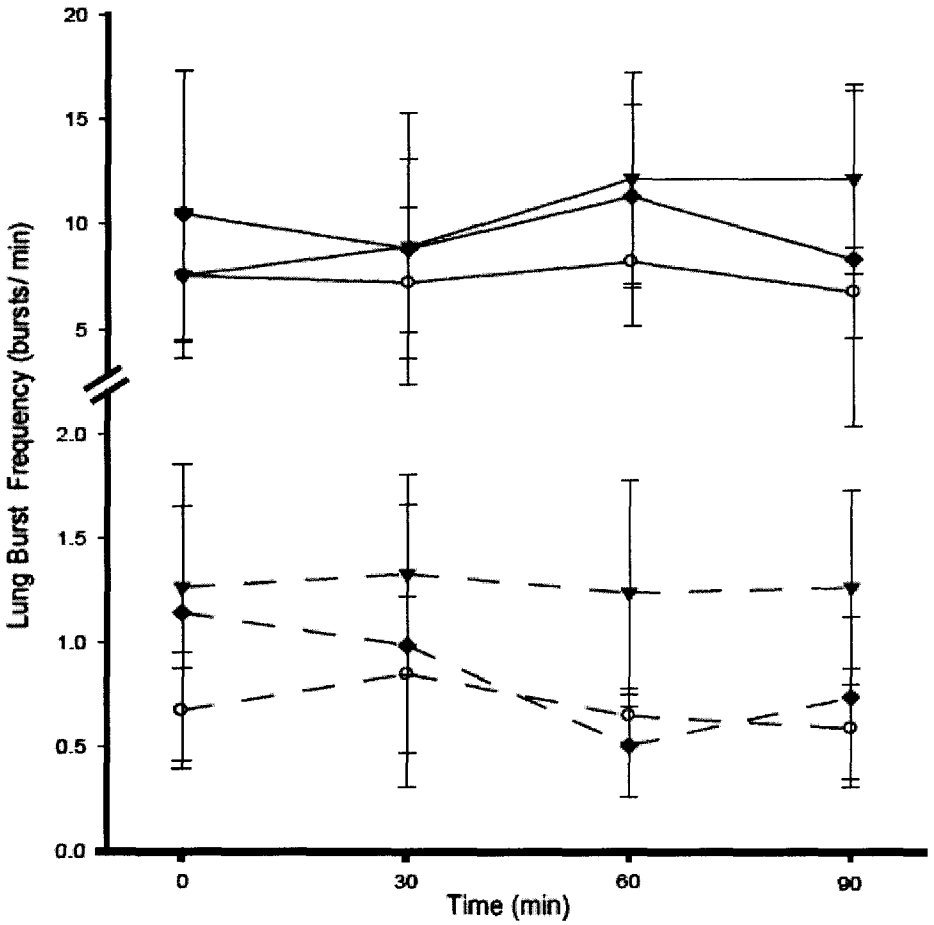

Fig 2.1 Time-control data on lung burst frequency of control and chronic ethanol-exposed tadpoles. Mean lung burst frequency over 3-min periods throughout normocapnic treatment. Neither early metamorphic (dashed lines) nor late metamorphic (solid lines) tadpoles significantly changed lung burst frequency over 90 min of normocapnia. Neither 3 wk (triangle) nor 10 wk (diamond) of ethanol exposure significantly changed lung burst frequency relative to controls (circle) Data presented are means ± SE for 7 - 8 tadpoles; $p > 0.05$ for all.

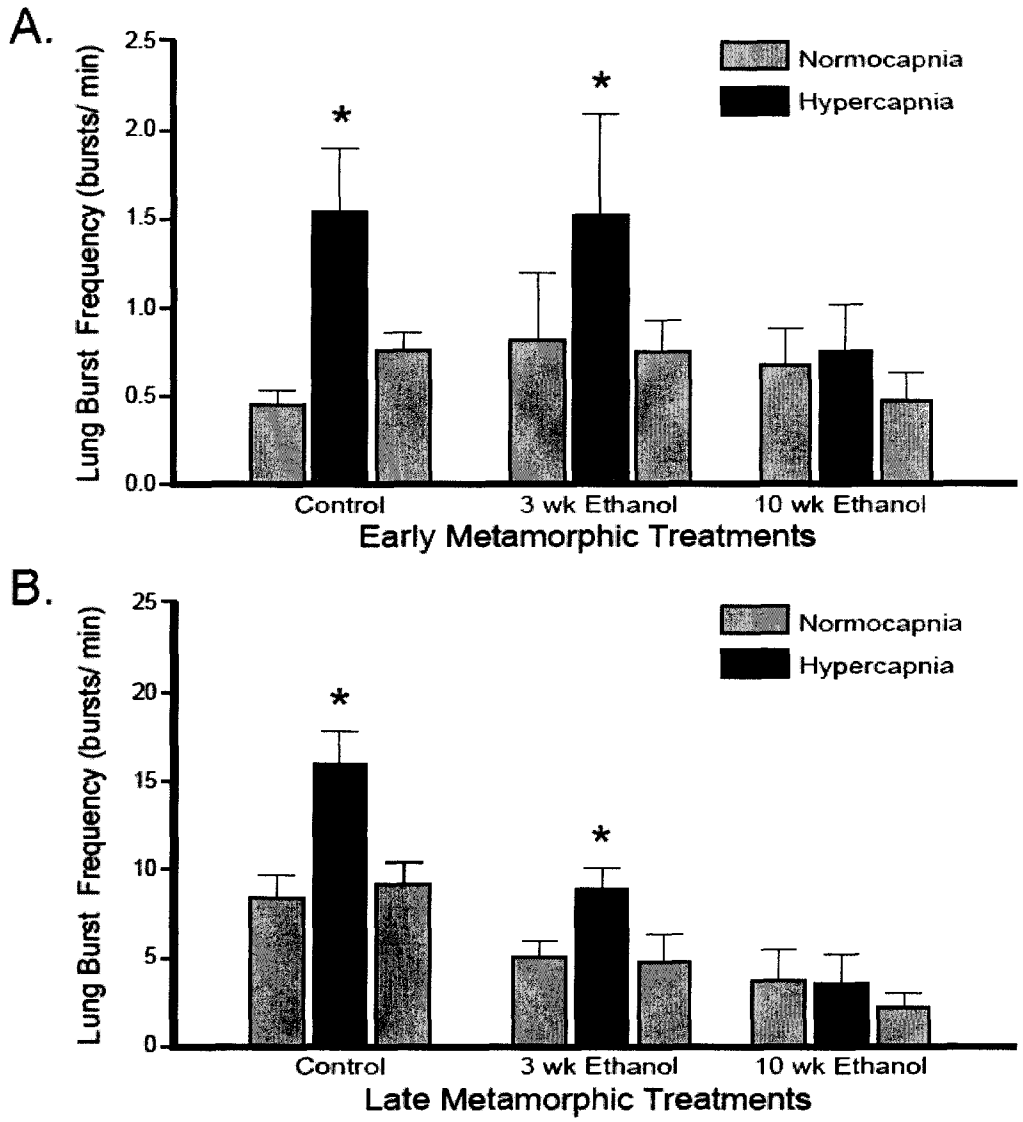

Fig 2.2 Effect of 3-wk and 10-wk ethanol exposure on tadpole central responses to hypercapnia. Mean lung burst frequency over last 3 min of 30-min normocapnia, hypercapnia, and normocapnic recovery in early (A) and late (B) metamorphic tadpoles. Both early and late metamorphic control and 3-wk ethanol-exposed tadpoles significantly increased lung burst frequency in response to hypercapnia (* = $p < 0.05$). Regardless of developmental stage, 10-wk ethanol-exposed tadpoles did not significantly change lung burst frequency during hypercapnia ($p > 0.05$). Data presented are means ± SE for 6 - 8 tadpoles.

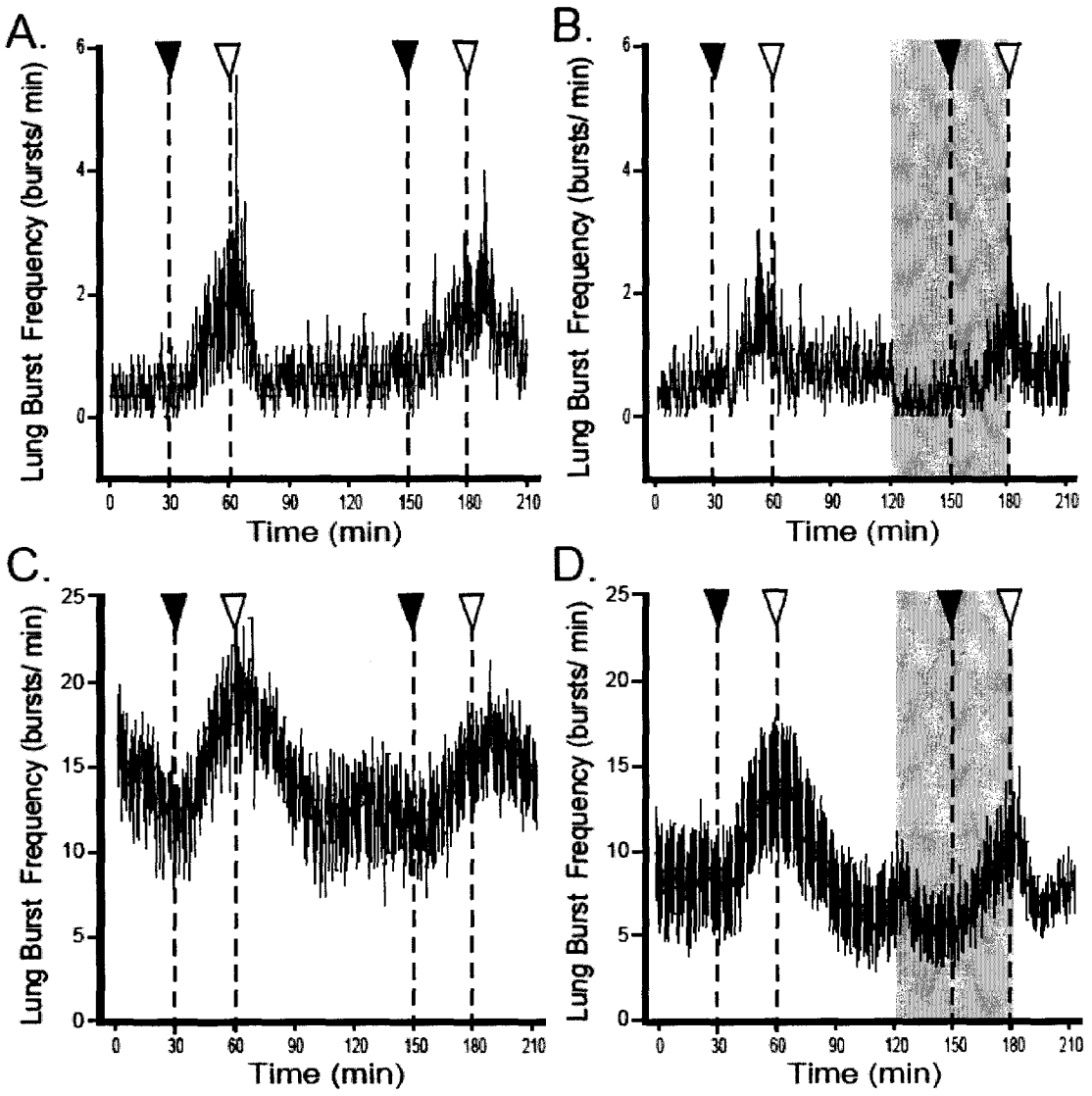

Fig 2.3 Acute ethanol exposure during the 210-min dual hypercapnic treatment protocol. Mean lung burst frequency per min, dashed lines signify a change in gas conditions from normocapnia to hypercapnia (black triangle) or hypercapnia to normocapnia (white triangle). Early (A) and late (C) metamorphic control tadpoles increased lung burst frequency during both hypercapnic treatments. Acute ethanol exposure (shaded region) depressed early metamorphic tadpole (B) lung burst frequency but did not inhibit response to hypercapnia. Late metamorphic tadpoles (D) were similar to controls; lung burst frequency during normocapnia and hypercapnia were unaffected by ethanol exposure. Data presented are mean ± SE for 6 - 8 tadpoles.

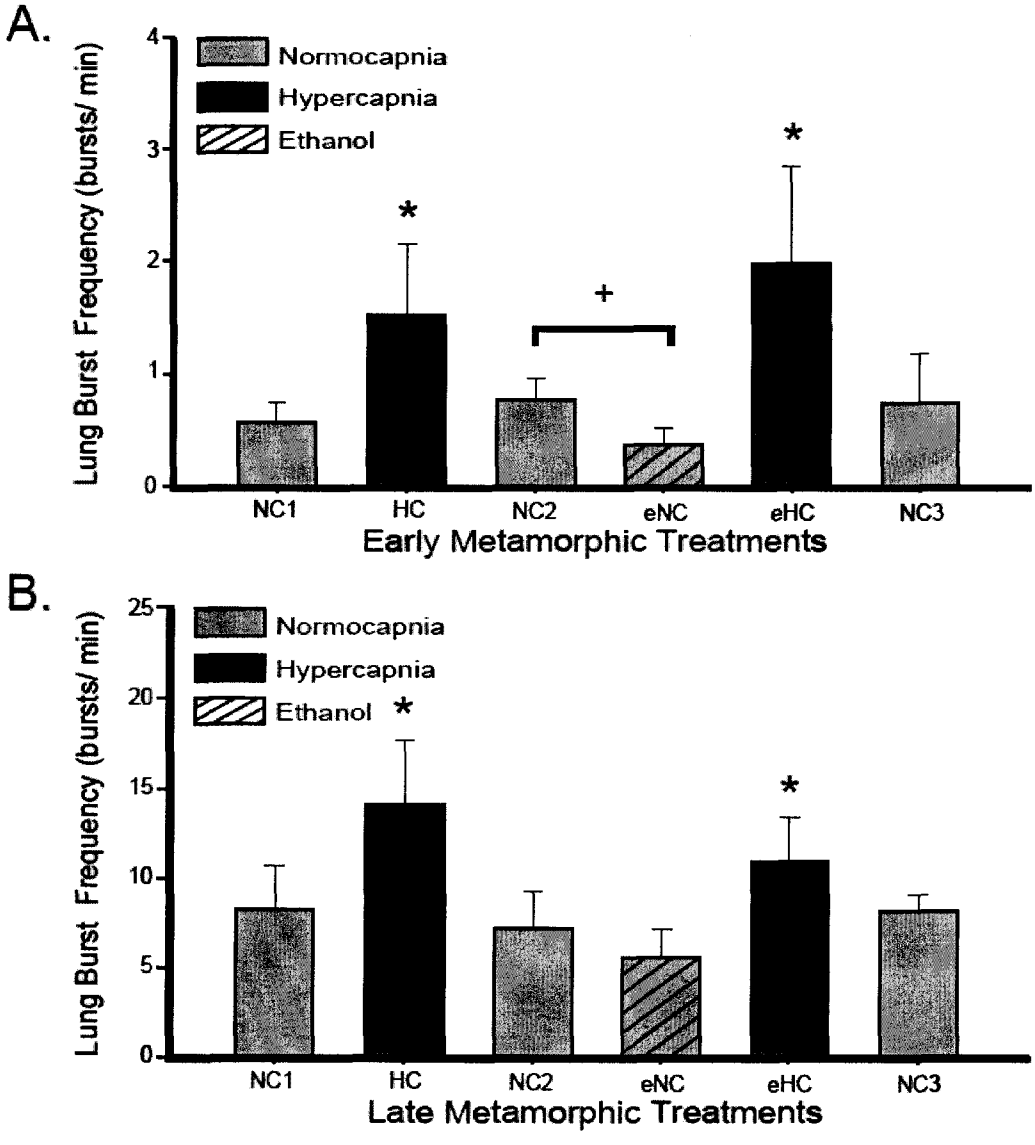

Fig 2.4 Effect of acute ethanol exposure on tadpole responses to hypercapnia. Mean lung burst frequency over the last 3 min of 30-min normocapnia (NC1), 30-min hypercapnia (HC), 60-min normocapnic recovery (NC2), 30-min acute ethanol normocapnia (eNC), 30-min acute ethanol hypercapnia (eHC), and 30-min normocapnic and ethanol-free recovery (NC3). Early metamorphic tadpoles (A) responded to hypercapnia with and without acute ethanol treatment (+ = $p < 0.05$), but their normocapnic lung neuroventilation was significantly decreased (* = $p < 0.01$). Neither the normocapnic nor hypercapnic lung burst frequency of late metamorphic tadpoles (B) was altered significantly by acute ethanol exposure. Data presented are means ± SE for 6- 8 tadpoles.

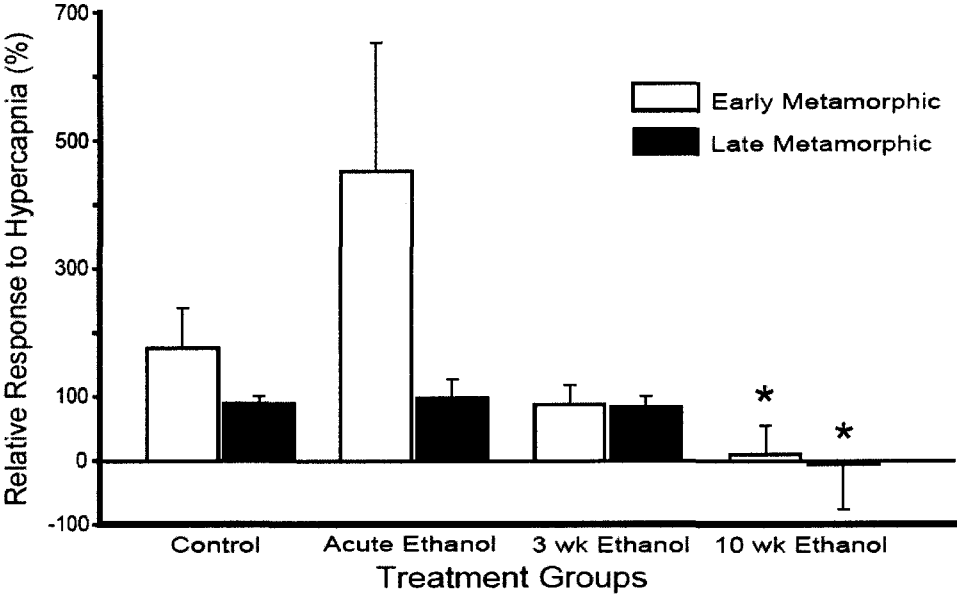

Fig 2.5 Hypercapnia-induced change in lung burst frequency in all control and ethanol treatment groups. Percent change in lung burst frequency (based on the mean during the last 3 min) from normocapnia, during hypercapnia for each treatment group. Responses to hypercapnia were statistically similar across development and between control, acute, and 3-wk ethanol-exposure groups ($p > 0.05$). Early and late metamorphic 10-wk ethanol-exposed tadpoles had a significantly lower relative response to hypercapnia (* = $p < 0.05$). Data presented are means ± SE for 6 - 8 tadpoles.

CHAPTER THREE

Chronic nicotine and ethanol exposure both disrupt central ventilatory responses to hypoxia in bullfrog tadpoles[1]

3.1 Abstract

Central hypoxic ventilatory response (HVR) consists of a reduction in ventilatory activity that follows a peripherally mediated ventilatory augmentation. Chronic early developmental nicotine and ethanol exposure are both impair the peripherally mediated HVR; nicotine impairs the central HVR, but the effect of ethanol on the central HVR has not been investigated. Additionally, chronic nicotine and ethanol exposure are known to impair ventilatory responses to hypercapnia in bullfrog tadpoles but HVRs were not tested. Here, early and late metamorphic tadpoles were exposed to either 30 μg/L nicotine or 0.12 - 0.06 g/dL ethanol for 10 wk. Tadpole brainstems were then isolated, and the neurocorrelates of ventilation were monitored *in vitro* over 180 min of hypoxia (PO_2 = 5.05 ± 1.04 kPa). Nicotine exposure reduced the normoxic

[1]Brundage, C.M., McLane, L.H. and Taylor, B.E. 2010. Chronic nicotine and ethanol exposure both disrupt central ventilatory responses to hypoxia in bullfrog tadpoles.(formatted for submission to) Journal of Physiology (London).

gill burst frequency of both early and late metamorphic tadpoles. Normoxic ventilation was unaffected by ethanol exposure. Both nicotine and ethanol disrupted central HVR. Nicotine impairments were dependent upon development. Central HVRs were impaired in only early metamorphic nicotine-exposed tadpoles. Both early and late metamorphic ethanol-exposed tadpoles failed to exhibit central HVRs. Thus, central HVRs are impaired following both nicotine and ethanol exposure

3.2 Introduction

Breathing is a vital behavior controlled by a brainstem neural network that must be responsive to respiratory stressors like hypercapnia and hypoxia. The ability to modulate breathing in response to hypoxia is characteristic of all vertebrate classes (McKenzie & Taylor, 1996). Prenatal exposures to the neuroteratogens, nicotine or ethanol, have been found to elicit functional impairments in the control of breathing and ventilatory responses to hypoxia (Smith *et al.*, 1991; Simakajornboon *et al.*, 2004; Dubois *et al.*, 2008; Campos *et al.*, 2009). Functional impairments in the breathing responses of neonates results often in

infant morbidity, mortality, and may be associated with sudden infant death (Hunt *et al.*, 1981; Kotagal, 2003; Cohen & Katz-Salamon, 2005; Bavis & Mitchell, 2008). Nicotine or ethanol exposure during early development are both associated with an increased risk of Sudden Infant Death Syndrome (SIDS; Haglund & Cnattingius, 1990; Feng, 1993; Iyasu *et al.*, 2002; Duncan *et al.*, 2008).

In mammals the hypoxic ventilatory response (HVR) consists of a peripherally mediated augmentation to compensate for low O_2 levels, followed by a ventilatory reduction that reflects a metabolic depression to adapt to low O_2 levels (Vizek *et al.*, 1987; Bisgard & Neubauer, 1995; Powell *et al.*, 1998). Most studies investigating the consequences of prenatal nicotine exposure on the HVR have focused on the augmentation phase (Milerad *et al.*, 1995; Bamford *et al.*, 1996; Ueda *et al.*, 1999). Simakajornboon and colleagues (2004) identified that both phases of the HVR are attenuated in 5-d rat pups following 15 d of continuous prenatal nicotine exposure. Prenatal ethanol exposure reduced baseline ventilation in juvenile rats and attenuated the peripherally mediated augmentation phase of the HVR (Dubois *et al.*, 2006; Dubois *et al.*, 2008). The effect of early developmental ethanol

exposure on the centrally mediated reduction phase of the HVR is however, currently unknown.

The developing bullfrog has been used as a model to investigate the ventilatory effects of nicotine and ethanol exposure (Taylor *et al.*, 2008; Brundage & Taylor, 2009; see chapter two). Use of bullfrogs is advantageous in providing for precise control of the conditions of exposure by eliminating maternal influences. The isolated brainstem preparation of the bullfrog produces a highly robust ventilatory motor output that has been used to study the control of breathing at all stages of development (Gdovin *et al.*, 1999; Harris *et al.*, 2002; Davies *et al.*, 2009). Eight to 12 wk of exposure to either 30 μg/L nicotine or 0.10 % ethanol (v/v) disrupts tadpole ventilatory responses to hypercapnia, both *in vivo* and in isolated brainstem preparations, without affecting baseline ventilation (Taylor *et al.*, 2008). Both the duration and timing of teratogen exposure are determining factors in the nicotine-, but not the ethanol-induced, hypercapnic impairments; early metamorphic tadpoles show increased vulnerability to nicotine (Brundage & Taylor, 2009; see chapter two). The aim of this study was to determine if the ventilatory

impairments elicited by developmental nicotine and ethanol exposure extend to the bullfrog tadpole central HVR.

Early metamorphic tadpoles initially increase gill and lung activity in response to moderate hypoxia (PO_2 ~100 mmHg; West & Burggren, 1982), but studies have not investigated the tadpole response to hypoxia that persists *in vivo* beyond the augmentation phase. The augmentation phase in lung ventilation during hypoxia becomes more dominant as metamorphosis progresses and tadpoles transition from obligate water to obligate air breathers (Burggren & Doyle, 1986). In the absence of peripheral afferents the isolated brainstems of bullfrog tadpoles demonstrate only the reduction phase of the HVR (Winmill *et al.*, 2005). Winmill and colleagues (2005) found that early metamorphic tadpoles are significantly more hypoxia tolerant than later developmental stages; reductions in lung neuroventilation are seen following 180 min of severe hypoxia (PO_2 near 0 kPa). Responding to similar conditions, later metamorphic tadpole stages show an earlier reduction in lung neuroventilation to the point of cessation within 30 min of severe hypoxia (Winmill *et al.*, 2005). There is some discrepancy among studies of the tadpole central HVR. Fournier and colleagues (2007) investigated

responses to 10-min hypoxia and identified an increase in lung burst frequency in early metamorphic tadpole preparations and a reduction in late metamorphic tadpole preparations. It is possible that amphibians are unique in that they retain some central involvement in the augmentation phase of the HVR early in development. Both studies are consistent in that neither found significant effects of hypoxia on gill neuroventilation (Winmill et al., 2005; Fournier et al., 2007). Thus, the central HVR of bullfrog tadpoles can be generally characterized as a change in lung burst frequency with persistent hypoxia.

We hypothesized that 10 wk of nicotine and ethanol exposure would both impair the central HVR of bullfrog tadpoles. Simakajornboon and colleagues (2004) reported central HVR impairment in mammals prenatally exposed to nicotine. Ours is the first study to evaluate the consequences of ethanol exposure on central HVRs. We expected that the teratogen impairment of the tadpole neuroventilatory response to one respiratory challenge, hypercapnia (Taylor *et al.*, 2008; see chapter two), would extend to another challenge, hypoxia. We assessed changes in neuroventilatory activity in both early and late metamorphic tadpoles to determine if developmental changes in hypoxia tolerance contribute to

the degree of central HVR impairment evoked by nicotine or ethanol exposure.

3.3 Methods

Animals

Studies were performed on *Lithobates* (formerly *Rana*) *catesbeiana* tadpoles (n = 39) purchased from a commercial supplier (Sullivan Co. Inc., www.researchamphibians.com). Tadpoles were maintained at room temperature and fed goldfish food daily. Tadpoles were housed for 10 wk in aquaria of either dechlorinated water only, dechlorinated water containing nicotine (30 μg/L (–)-nicotine hydrogen tartrate salt; Sigma, www.sigmaaldrich.com) or dechlorinated water containing ethanol (0.12 - 0.06 g/dL). The concentrations of nicotine and ethanol were consistent with those of a previous study using bullfrogs (Taylor *et al.*, 2008). The nicotine concentration was based on that found in the body fluids of average smokers (Moyer *et al.*, 2002). The ethanol concentration varied due to the volatilization from the aquarium water and was equivalent to 0.75 - 1.5 times the 0.08 g/dL blood alcohol content that is the legal

limit of many western countries (Caldeira *et al.*, 2004). We chose 10-wk exposure because that duration of exposure impairs ventilatory responses to hypercapnia in both early and late stage tadpoles (Taylor *et al.*, 2008; Brundage & Taylor, 2009; see chapter two). We sought to determine if responses to hypoxia were affected after the same 10-wk teratogen exposures.

The developmental stage of each tadpole was determined twice, once at the start of treatment and again at the time of dissection to ensure developmental homogeneity of the treatment groups. At the time of dissection each tadpole was either "early metamorphic" (forelimbs absent, hind limbs paddle-like without joints or separated toes) or "late metamorphic" (forelimbs and hind limbs present, tail being resorbed), corresponding to developmental stages 7 - 10 or 19 - 25, respectively, in the classification scheme of Taylor and Köllros (1946). This was true for animals that received either 3 or 10 wk of chronic teratogen exposure, as they were all maintained for 10 wk. Animals that were not within these stage ranges after 10 wk were excluded from the datasets. Tadpoles included in this study consisted of 19 early metamorphic tadpoles (6 control, 7 nicotine-, and 6 ethanol-exposed) and 20 late metamorphic

tadpoles (6 control, 6 nicotine-, and 8 ethanol-exposed). All animal care and experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Alaska Fairbanks and complied with all state and federal ethical guidelines

Surgical preparation

Each tadpole was anesthetized by immersion for 1 - 2 min in cold (4 °C) 0.2 mM tricaine methanesulfonate (MS222; Sigma, www.sigmaaldrich.com) in dechlorinated water buffered to pH 7.8 with $NaHCO_3$. The front of the head rostral to the nares and the back of the body (hind limbs and tail, if present) were removed. The dorsal cranium and forebrain rostral to the diencephalon were resected and the fourth ventricle opened by removing the choroid plexus. The remaining brainstem and spinal cord were removed *en bloc* and further trimmed rostrally to the optic tectum and caudally to the brachial nerve. During dissection, exposed tissues were superfused with cold artificial cerebral spinal fluid (aCSF) composed of (in mM) 104 NaCl, 4 KCl, 1.4 $MgCl_2$, 10 D-glucose, 25 $NaHCO_3$ and 2.4 $CaCl_2$ equilibrated with 100 % O_2. This single aCSF composition has been used in previous studies of

neuroventilation in bullfrog tadpoles and juveniles (Taylor *et al.*, 2003a; 2003b; 2008) and was employed here to ensure comparability with those studies and between experiments on animals of different metamorphic stages.

The isolated brainstem-spinal cord was transferred *en bloc* to a 2.5 ml, Plexiglas, flow-through recording chamber and was supported, ventral side up, between coarse nylon mesh such that all surfaces were bathed with aCSF flowing from rostral to caudal at a rate of 5 ml/min. A supply of aCSF, equilibrated with O_2-CO_2 mixtures, flowed through plastic tubing to the recording chamber and bathed the isolated brainstem. CO_2 was monitored with a CO_2 analyzer (Capstar 100; CWE, www.cwe-inc.com). The pH of the aCSF was maintained at pH 7.8 (~9 torr PCO_2) by adjusting the fractional concentration of CO_2 in the equilibration gas. After isolation the brainstem was allowed to stabilize for 1 h while superfused at 23 °C.

Nerve recording

Roots of the facial and hypoglossal nerves were drawn into glass suction electrodes pulled from 1 mm diameter capillary glass to tip diameters that fit the nerve roots. Whole-nerve discharge was amplified (X100 by DAM 50 amplifiers, World Precision Instruments, www.wpiinc.com; X1000 by a four-channel model 1700 amplifier, A-M Systems, www.a-msystems.com) and filtered (100 Hz high pass to 1 kHz low pass). The amplified and filtered nerve output was sent to a data acquisition system (Powerlab, AD Instruments, www.adinstruments.com), which sampled at 1 kHz, data were archived as whole-nerve discharge, and duplicate integrated (full-wave rectified and averaged over 200 ms) neurograms were acquired simultaneously. Such recordings were made during the initial 1-h post-isolation stabilization period and recorded continuously throughout the duration of each treatment protocol.

Hypoxia treatment

After the stabilization period the aCSF brainstem perfusate remained at pH 7.8 with the equilibrated O_2-CO_2 mixtures for an additional 30 min to

establish normoxic baseline neuroventilation. Then the O_2 gas was replaced with N_2 while CO_2 levels remained constant. The N_2/CO_2 equilibrated aCSF (hypoxia) bathed the tissue at a flow rate of 5-7 ml/min for 180 min. After the hypoxia treatment the N_2 was replaced with O_2 for 30 min of normoxic recovery. The aCSF remained isocapnic throughout the entire treatment protocol. The hypoxia treatment and duration is similar to that employed in other bullfrog studies (Winmill *et al.*, 2005; Fournier *et al.*, 2007) and differs only by our use of a single aCSF reservoir rather than separate reservoirs for normoxic and hypoxic aCSF. Using an oxygen electrode (MI-730, Microelectrodes Inc., www.microelectrodes.com) the PO_2 in the recording chamber of our apparatus was evaluated; within 10 min of switching to hypoxic equilibration of the aCSF reservoir, the recording chamber reached 5.05 ± 1.04 kPa of O_2. There was no subsequent change in PO_2 over the 180 min of hypoxia treatment.

Data and statistical analyses

Neurograms recorded from the isolated brainstems were quantified for 30 min of normoxia, 180 min of hypoxia, and for 30 min of subsequent

return to normoxia. Burst activity patterns were designated as either putative gill or putative lung breaths on the basis of the amplitude of the integrated nerve activity and the presence or absence of coincident firing in both the facial and hypoglossal nerves as previously described (Torgerson *et al.*, 1998). Putative gill breaths had lower integrated burst amplitude on the facial nerve than putative lung breaths, and little or no coincident burst activity in the hypoglossal nerve. Putative lung breaths had higher integrated burst amplitude in the facial nerve and coincident burst activity in the hypoglossal nerve.

The frequency of lung ventilation was quantified as the mean number of lung bursts per minute over 10-min intervals of normoxia and hypoxia. Each animal's peak hypoxic response was calculated as the lowest 10-min lung burst frequency during the 180 min of hypoxia. The change in lung burst frequency for each 10 min of hypoxia relative to the last 10 min of baseline was used to quantify treatment group responses over time. The duration and amplitude of putative lung and gill bursts as well as the frequency of gill bursts were quantified for the last 3 consecutive minutes of normoxic baseline, the 10-min intervals ending at 10, 60, 90, 120, 150, and 180 min of hypoxia, and normoxic recovery. The mean

values for each of the quantified neuroventilatory parameters were compared using repeated-measures analysis of variance (RM-ANOVA; SigmaStat, www.systat.com). When an RM-ANOVA indicated that significant differences existed, multiple comparisons were made using the Holm-Sidak multiple comparison test. Comparisons between early and late metamorphic tadpoles, and between control and treatment groups during normoxia, were made using t-test comparisons (SigmaStat, www.systat.com). All values reported in the text are means ± SE. The lowest P value is listed in instances when more than three non-significant values are reported.

3.4 Results

Effect of chronic nicotine or ethanol exposure on early metamorphic tadpole lung neuroventilation

Control tadpole brainstems exhibited decreased lung burst frequency in response to hypoxia. Early metamorphic tadpoles reduced lung burst frequencies from 0.52 ± 0.12 to 0.09 ± 0.04 bursts/min (P = 0.010; Fig. 3.1A); this reduction took place late in hypoxia with a significant percent

change from baseline at 170 and 180 min of hypoxic exposure (P = 0.011 and P = 0.046, respectively; Fig. 3.1B), and lung burst frequency recovered fully within 30 min of return to normoxia (15.04 ± 47.88 %).

Early metamorphic tadpoles exposed to nicotine for 10 wk had a mean lung burst frequency of 1.26 ± 0.61 burst/min, which was not significantly different from control tadpoles (P = 0.291; Fig. 3.1A). In response to hypoxia, early metamorphic nicotine-exposed tadpoles did lower lung burst activity, but not significantly (0.21 ± 0.07 bursts/min; P = 0.116). The percent change in lung burst frequency from baseline showed an early drop, but did not change significantly over the course of hypoxic exposure (P = 0.980; Fig. 3.1B).

Early metamorphic tadpoles exposed to ethanol for 10 wk had a mean lung burst frequency of 0.63 ± 0.24 burst/min, which was not significantly different from control tadpoles (P = 0.680). Mean of the animals' lowest 10-min period of lung neuroventilation was not depressed significantly during hypoxia although it was lower than baseline (0.10 ± 0.04 burst/min; P = 0.056; Fig. 3.1A). The percent change in lung burst frequency from baseline in chronic ethanol-exposed

early metamorphic tadpoles did not vary significantly over the course of hypoxic exposure (P = 0.997; Fig. 3.1B). Thus, 10 wk of either nicotine or ethanol exposure disrupted the early metamorphic tadpole central HVR.

Neither the duration (Fig. 3.2A) nor the amplitude (Fig. 3.2B) of lung bursts varied significantly during hypoxia in early metamorphic tadpoles (P = 0.675 and 0.939, respectively). Neither nicotine nor ethanol exposure affected the amplitude or duration of lung bursts in early metamorphic tadpoles, and neither teratogen altered these burst parameters significantly during hypoxia (all P values ≥ 0.356). Therefore, lung burst frequency, the only burst parameter affected by hypoxia, was altered following 10-wk nicotine or ethanol exposure.

Effect of chronic nicotine or ethanol exposure on late metamorphic tadpole lung neuroventilation

The lung burst frequency of late metamorphic tadpoles was significantly greater than early metamorphic tadpoles (9.39 ± 2.83 bursts/min; P = 0.011). In response to hypoxia, brainstems from control late metamorphic tadpoles significantly reduced lung burst frequencies to

3.39 ± 0.64 bursts/min (P = 0.032; Fig. 3.3A). In contrast to early metamorphic tadpoles, late metamorphic tadpoles demonstrated a more rapid reduction in lung burst frequency; a significant percent decrease was apparent after 60 min of hypoxia and extended throughout the hypoxia treatment except around110 min ($P < 0.05$ for all; Fig. 3.3B). Lung burst frequency had recovered from the hypoxia-induced decrease by 30 min of normoxia, no longer exhibiting a significant percent change from baseline normocapnia (P = -17.98 ± 20.64 %).

The lung burst frequency of late metamorphic tadpoles following 10 wk of nicotine exposure was elevated, but not significantly different from control tadpoles (13.80 ± 9.98 bursts/min; P = 0.388; Fig. 3.3A). In response to hypoxia, this lung burst frequency decreased significantly to 2.34 ± 0.82 bursts/min (P = 0.036). Significant reductions in lung burst frequency exhibited by nicotine-exposed late metamorphic tadpoles occurred at 100 - 130 min and 150 - 180 min of hypoxia ($P < 0.001$; Fig. 3.3B), which is later than the 60-min response time of control late metamorphic animals. Following 30 min of normoxic recovery, the lung burst frequency recovered to a level not significantly different from baseline (-31.23 ± 18.44 %).

Ten wk of ethanol exposure did not significantly alter the normoxic lung burst frequency of late metamorphic tadpoles (3.69 ± 1.16 bursts/min; P = 0.074; Fig. 3.3A) although this frequency was less than controls. The lowest 10-min interval of lung neuroventilation during hypoxia, though lower, was not significantly different from baseline (1.54 ± 0.54 burst/min; P = 0.109). Hypoxia had no significant effect on the lung burst frequency of 10-wk ethanol-exposed late metamorphic tadpoles through the duration of hypoxic exposure (P = 0.85; Fig. 3.3B). Thus, 10 wk of ethanol, but not nicotine, exposure disrupts the central HVR of late metamorphic tadpoles.

Neither the duration (Fig. 3.4A) nor the amplitude (Fig. 3.4B) of lung bursts varied significantly during hypoxia in late metamorphic tadpoles (P = 0.648 and 0.396, respectively). Neither nicotine nor ethanol exposure affected the amplitude or duration of lung bursts in late metamorphic tadpoles, nor did either teratogen significantly alter these burst parameters during hypoxia (all P values ≥ 0.220). It is interesting to note that the lung burst amplitude of ethanol-exposed late metamorphic tadpoles was elevated although not significantly during late hypoxia treatment. Ultimately, only those lung burst parameters

associated with late metamorphic tadpole responses to hypoxia were significantly altered following 10-wk ethanol but not nicotine exposure. Therefore, like early metamorphic tadpoles, lung burst frequency was the only lung neuroventilatory response parameter altered in 10-wk ethanol-exposed late metamorphic tadpoles, and unlike the early developmental group, 10-wk nicotine exposure had no effect on lung burst parameters during normoxia or hypoxia.

Effect of chronic nicotine or ethanol exposure on gill neuroventilation

Gill burst frequency of control early metamorphic tadpoles was not significantly different during normoxia and hypoxia (42.67 ± 3.72 bursts/min; P = 0.287; Fig. 3.5). Furthermore, this gill burst frequency was not significantly different from late metamorphic tadpoles (35.57 ± 2.31 bursts/min; P = 0.122). The gill frequency of late metamorphic tadpoles did increase during hypoxia but not consistently. The increases occurred only around 60 and 90 min of hypoxic exposure (42.57 ± 2.45 bursts/min; P = 0.028 and 44.50 ± 2.72 bursts/min; P = 0.050 for 60 and 90 min of hypoxia, respectively; Fig. 3.5C).

Compared to control tadpoles 10 wk of nicotine exposure had a considerable effect on gill burst frequency. The gill burst frequencies of early and late metamorphic 10-wk nicotine-exposed tadpoles were significantly different from one another ($P = 0.014$) and both were significantly less than that of control animals (28.10 ± 1.64 bursts/min; $P = 0.003$ and 18.78 ± 2.86 bursts/min; $P < 0.001$ for early and late 10-wk nicotine-exposed tadpoles, respectively). Similar to controls, the gill burst frequency of early metamorphic nicotine-exposed tadpoles was unchanged during hypoxia ($P = 0.267$), but the gill frequency of late metamorphic tadpoles increased briefly around 90 and 120 min of hypoxia (30.39 ± 3.87 bursts/min; $P = 0.021$ and 28.44 ± 3.21 bursts/min; $P = 0.012$ for 90 and 120 min of hypoxia, respectively).

The normoxic gill burst frequencies of early and late metamorphic 10-wk ethanol-exposed tadpoles was not significantly different from those of controls (34.83 ± 4.50 bursts/min; $P = 0.209$ and 39.29 ± 4.03 bursts/min; $P = 0.440$ for early and late metamorphic tadpoles, respectively) nor did they differ significantly from each other ($P = 0.475$). Unlike controls, the gill burst frequency of early metamorphic ethanol-

exposed tadpoles increased by 90 min of hypoxia and remained elevated for the duration of the hypoxic exposure ($P = 0.039$). This gill frequency remained significantly increased above baseline levels despite 30 min of normoxic recovery (40.67 ± 3.7 bursts/min; $P = 0.013$). Late metamorphic 10-wk ethanol-exposed tadpoles also differed from controls in that they did not exhibit a significant change in gill burst frequency throughout the course of hypoxic exposure ($P = 0.593$).

Gill burst frequency is closely linked with burst duration. Gill burst duration did not change significantly either in response to hypoxia or following exposure to nicotine or ethanol (all P values ≥ 0.203; Fig. 3.6). Early metamorphic nicotine-exposed tadpoles did demonstrate a transient increase in gill duration; however, this change was not significant and did not offset the lack of gill burst frequency change in that treatment group ($P = 0.307$). Thus, nicotine exposure alters baseline gill burst frequencies without affecting gill burst frequency responses to hypoxia, and ethanol exposure altered gill burst frequency responses to hypoxia without changing baseline gill burst activity.

In control tadpoles the amplitude of gill bursts increased significantly with development from 0.10 ± 0.02 V in early metamorphic tadpoles to 0.25 ± 0.05 V in late metamorphic tadpoles ($P = 0.039$). Hypoxia, however, did not have a significant effect on gill burst amplitude in either developmental group ($P = 0.78$ and $P = 0.401$ for early and late metamorphic tadpoles, respectively; Fig. 3.7). 10 wk of nicotine exposure did not significantly alter the normoxic gill burst amplitude of either developmental group (0.11 ± 0.03 V; $P = 0.701$ and 0.17 ± 0.04 V; $P = 0.229$ for early and late metamorphic 10-wk nicotine-exposed tadpoles, respectively), and the gill burst amplitudes of early and late metamorphic nicotine-exposed tadpoles were not significantly different from each other ($P = 0.312$). In response to hypoxia, the gill burst amplitude of early metamorphic tadpoles did not change significantly during the course of hypoxia ($P = 0.568$). Late metamorphic tadpoles, however, demonstrated a significant reduction in gill amplitude at 150 and 180 min of hypoxia (0.08 ± 0.02 V; $P = 0.043$ and 0.08 ± 0.03; $P = 0.031$, respectively). Within this developmental group, gill burst amplitude remained significantly reduced from baseline levels following 30 min of normoxic recovery (0.09 ± 0.03 V; $P = 0.046$).

Ten wk of ethanol exposure did not significantly alter the normoxic gill burst amplitude of either early or late metamorphic tadpoles relative to that of control tadpoles (0.12 ± 0.24 V; P = 0.552 and 0.15 ± 0.04 V; P = 0.180, respectively), and the gill burst amplitudes of early and late metamorphic ethanol-exposed tadpoles were not significantly different from each other (P = 0.480). Similar to controls the gill burst amplitude of both early and late metamorphic ethanol-exposed tadpoles did not vary significantly over the 180 min of hypoxia (P = 0.196 and P = 0.891 for early and late metamorphic tadpoles, respectively).

In summary, early and late metamorphic normoxic gill burst frequency and the late metamorphic hypoxic gill burst amplitude were affected by 10 wk of nicotine exposure. Ten-wk ethanol exposure did not affect normoxic gill neuroventilation; however, during hypoxia the gill burst frequency of ethanol-exposed animals was markedly changed.

3.5 Discussion

Ten-wk exposure to either nicotine or ethanol impaired tadpole central HVRs (hypoxic ventilatory responses). The tadpole central HVR is a

reduction in lung burst frequency as seen in control tadpoles. Late metamorphic tadpoles exhibited this reduction earlier in the hypoxic treatment than early metamorphic tadpoles, and in both metamorphic stages lung burst frequency recovered to baseline levels following 30 min of normoxia. Unlike lung ventilation which increases with tadpole development, gill ventilation remains relatively constant. Hypoxia had little effect on gill burst frequency. Only late metamorphic tadpoles demonstrated hypoxia-induced changes in gill burst frequency, transient increases around 60 and 90 min of hypoxia. Therefore, the tadpole central HVR manifests as a decrease in lung burst frequency and an ephemeral increase in late metamorphic tadpole gill burst frequency., The amplitude and duration of lung bursts, as well as the amplitude, and duration of gill bursts, were not altered in response to hypoxia.

Effect of nicotine on bullfrog tadpole neuroventilation

Early metamorphic tadpoles did not exhibit a central HVR following 10-wk nicotine exposure. There was an early attenuation in lung activity, however, neither the time course nor the degree of ventilatory depression were similar to control animals. Late metamorphic nicotine-exposed

tadpoles did exhibit decreased lung burst frequency, a central HVR, and an increase in gill burst frequency at 90 and 120 min of hypoxia. Both the lung burst frequency reduction and gill burst frequency augmentation in late metamorphic nicotine-exposed tadpoles occurred later in the hypoxic treatment than similar changes in controls. This delayed hypoxic response suggests increased hypoxia tolerance, decreased hypoxia sensitivity, or some hindrance to the hypoxic response. An increase in hypoxia tolerance is unlikely as nicotine also caused a reduction in gill burst amplitude late in hypoxia that did not recover during normoxia. These findings suggest that chronic nicotine exposure delays the central HVR by impairing the breathing control network's mechanisms for sensing and/or responding to hypoxia.

Loss of the central HVR following 10-wk nicotine exposure was dependent on the timing of that exposure coinciding with early development, which suggests that early metamorphic tadpoles are more vulnerable to the deleterious neural effects of chronic nicotine exposure. This finding was similar to those of studies looking at the effect of chronic nicotine exposure on hypercapnic ventilation, where early metamorphic tadpoles exhibited an impaired hypercapnic response after

3-wk nicotine exposure, whereas late metamorphic tadpoles required 10-wk exposure to exhibit the same impairment (Brundage & Taylor, 2009). The effects of acute nicotine exposure, however, are opposite in that they increase with bullfrog development (Brundage *et al.*, 2010). Maturation of the cholinergic system in the bullfrog brainstem and ontogeny of ventilatory chemoreflexes may play key roles in defining the period of nicotine vulnerability. Numerous studies have determined that prenatal nicotine exposure impairs the postnatal hypoxic response of rats and lambs (Slotkin *et al.*, 1995; Fewell & Smith, 1998; Fewell *et al.*, 2001b, a; Hafstrom *et al.*, 2002), but the effects of varying timing and duration of this developmental exposure have not been specifically investigated. In rat pups, prenatal nicotine exposure impairs the central HVR in an age-dependent manner; impairments are exhibited in the early postnatal days but not later than postnatal day 10 (Simakajornboon *et al.*, 2004). It remains to be seen if early metamorphic tadpoles can recover from nicotine-induced impairments of central ventilatory responses.

We have previously shown that 10 wk of nicotine exposure has no effect on normoxic/normocapnic lung neuroventilation nor does it affect the integrity of the isolated bullfrog brainstem lung activity over the duration

of our experiment (Taylor *et al.*, 2008; Brundage & Taylor, 2009). Here we see that 10-wk nicotine exposure did have a significant effect on gill burst activity. Nicotine-exposed tadpoles had significantly fewer gill bursts per min than control tadpoles of the same developmental group, and late metamorphic nicotine-exposed tadpoles had significantly fewer gill bursts than similarly exposed early metamorphic tadpoles. Gill amplitude was also reduced in late metamorphic nicotine-exposed tadpoles at the end of the hypoxia and did not recover, which suggests that chronic nicotine exposure may have disrupted hypoxia tolerance in these tadpoles rather than their central HVR. Early metamorphic tadpoles appear to be more vulnerable to a nicotine-induced impairment of the central HVR, but late metamorphic nicotine-exposed tadpoles appear to be more vulnerable to hypoxia given they were the only nicotine-exposed group to exhibit a hypoxia-induced neuroventilatory change that was not reversed during 30 min of normoxic recovery.

Effect of ethanol on bullfrog tadpole neuroventilation

This is the first study to investigate the effect of ethanol exposure on the central HVR. Ethanol exposure, regardless of developmental stage,

disrupted the tadpole central HVR. Neither early nor late metamorphic tadpoles that were chronically exposed to ethanol demonstrated a significant change in lung burst frequency over the 180-min hypoxia treatment. There was a slight depression in the lung burst frequency of late metamorphic ethanol-exposed tadpoles at 180 min, and it is possible that a significant reduction in lung burst frequency would occur if hypoxia was sustained longer. Late metamorphic ethanol-exposed tadpoles failed to increase gill burst frequency at any time during hypoxia; however, early metamorphic ethanol-exposed tadpoles exhibited an increased gill burst frequency during the latter half of hypoxia treatment.

All neuroventilatory parameters were consistent between ethanol-exposed and control tadpoles during normoxic conditions; this is similar to previous studies demonstrating that the integrity of ethanol-exposed isolated brainstem preparations is unaltered over the duration of our experiment (Taylor *et al.*, 2008; see chapter two). This would suggest that the central HVR was specifically impaired by 10-wk ethanol exposure. These results are different than those of semi-intact preparations derived from neonatal rats that had been prenatally exposed to ethanol, where

baseline ventilation and the HVR were reduced (Dubois *et al.*, 2008). It is possible that these differing results are due to chronic ethanol altering peripheral chemoreception and/or the presence of afferent signaling mechanisms which were present in the semi-intact rat preparations. Additional studies with either semi-intact tadpole preparations or isolated rat brainstem preparations would be required to clarify this point.

Consequences of nicotine and ethanol exposure on neural signaling

Signaling pathways that underlie the HVR in bullfrogs and mammals and pathways that are affected by chronic nicotine or ethanol exposure, to date, have been investigated separately. We can, however, make inferences about possible mechanisms of these teratogenic effects on the central HVR by jointly considering several independent studies. Fournier and colleagues (2007) determined that the bullfrog central HVR is modulated by noradrenergic neurons via an indirect GABAergic/glycinergic pathway. The α_1-antagonist prazosine blocked the central lung burst HVR in both early and late metamorphic tadpoles. Application of a bicuculine/strychine mixture blocked both the HVR and

the effect of the α_1-agonist phenylephrine in isolated brainstem preparations. In rats, prenatal nicotine exposure alters both GABAergic and glycinergic signaling in the Pre-Bötzinger complex (Luo *et al.*, 2007), a putative respiratory rhythm generator. Nicotinic acetylcholine receptors are up-regulated on GABAergic and glycinergic neurons, and chronic stimulation of these neurons results in desensitization and a reduction in GABA and glycine release (Covernton & Lester, 2002; Neff *et al.*, 2003; Fregosi & Pilarski, 2008). In bullfrog tadpoles, a reduction in inhibitory signaling may contribute to the loss of the central HVR in early metamorphic tadpoles. Similarly, ethanol is a known GABAmimetic and imidazobenzodiazepine $GABA_A$ receptor agonist (Breese *et al.*, 2006; Wallner & Olsen, 2008). Prenatal ethanol exposure has also been shown to alter GABA transmission and GABAergic neuron activity (Janiri *et al.*, 1994; Allan *et al.*, 1998). An increase in central glycinergic tonic inhibition is also seen following prenatal ethanol exposure (Dubois *et al.*, 2008). Binding characteristics and ligand specificity of $GABA_A$ receptors are similar in bullfrogs and mammals (Hollis & Boyd, 2003). Chronic ethanol may, therefore, generate similar disruptions in GABAergic and glycinergic activity in both early and late metamorphic tadpoles contributing to a loss of the central HVR. Thus, existing evidence

suggests that the central HVR and the effects of chronic nicotine and ethanol exposure overlap in their links to GABAergic and glycinergic signaling pathways but not to the exclusion of other possibilities.

If chronic nicotine and ethanol exposure exert their deleterious effects on control of breathing via a common mechanism, GABAergic signaling is a potential candidate. The mechanism by which nicotine and ethanol impair the central HVR, however, need not be a common pathway. Prenatal nicotine has been found to influence serotonergic, glutamatergic, and adrenergic signaling (Campos *et al.*, 2009). Ethanol has both direct and indirect effects on neural tissue including the desensitization of multiple ionotropic receptor subtypes (Aguayo *et al.*, 2002; Dopico & Lovinger, 2009). Identifying the multifarious consequences of developmental nicotine and ethanol exposure remains the subject of on-going research as do the multiplicity of sites and heterogeneity of neural mechanisms involved in the control of breathing.

The central hypoxic ventilatory response of bullfrog tadpoles

The central HVR of bullfrog tadpoles differed slightly in our study from those previously conducted. The central HVR we found in early metamorphic tadpoles was similar to Winmill and colleagues (2005) who identified a reduction in lung burst frequency late in hypoxia treatment. Fournier and colleagues (2007) described an increase in early metamorphic lung burst frequency at 10 min of hypoxia exposure suggesting some central involvement in the augmentation phase of the HVR exhibited by intact tadpoles. We saw no such increase; however, lung activity of early metamorphic tadpoles fluctuated prior to the reduction in lung burst frequency. It is possible that individual variation precluded our ability to identify a significant increase. In all three studies the central HVR of later metamorphic stages was a reduction in lung burst frequency (Winmill *et al.*, 2005; Fournier *et al.*, 2007). Fournier and colleagues (2007) reported a significant reduction at 10 min hypoxia, Winmill (2005) reported a cessation of lung activity by 30 min of hypoxia, and in our hands lung burst frequency was significantly reduced after 60 min of hypoxia. Variation in the time course and degree of reduction in lung burst frequency may be due to the onset speed and degree of

hypoxic insult employed in each study. In our study the onset of the hypoxia was slower (10 min vs. 5 min) and the degree of hypoxia was less (5.05 ± 1.04 kPa vs. near 0 kPa; Winmill *et al.*, 2005). Despite differences in technique, we recorded central HVRs in bullfrog tadpoles that were similar to previously reported responses. Furthermore, we demonstrated that the central HVR was impaired in 10-wk ethanol-exposed tadpoles of either developmental group and early metamorphic 10-wk nicotine-exposed tadpoles.

The ability to respond to hypoxia is a fundamental feature of vertebrate ventilation (McKenzie & Taylor, 1996). Ten wk of nicotine exposure induced a development-dependent difference in gill burst frequency during normoxia and impaired the central HVR of early but not late metamorphic tadpoles. Ten wk of ethanol exposure impaired the central HVR of both early and late metamorphic tadpoles without affecting normoxic neuroventilation, gill or lung. Evidence presented here and by others suggests that developmental exposure to either nicotine or ethanol impairs both peripherally and centrally mediated responses to hypoxia in amphibians and mammals.

3.6 Acknowledgments

Ms. Lisa H. McLane assisted in analyzing control and ethanol treatment data. Dr. Barbara E. Taylor served as my advisor and was the principal investigator for the laboratory overseeing this research. We would like to thank Mr. Justin Buehner for his contribution to the dataset used in this study and Ms. Carla Nelson for her assistance analyzing nicotine treatment data. This work was supported by NIH-NINDS grant # U54 NS041069-06A1.

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3.8 Figures

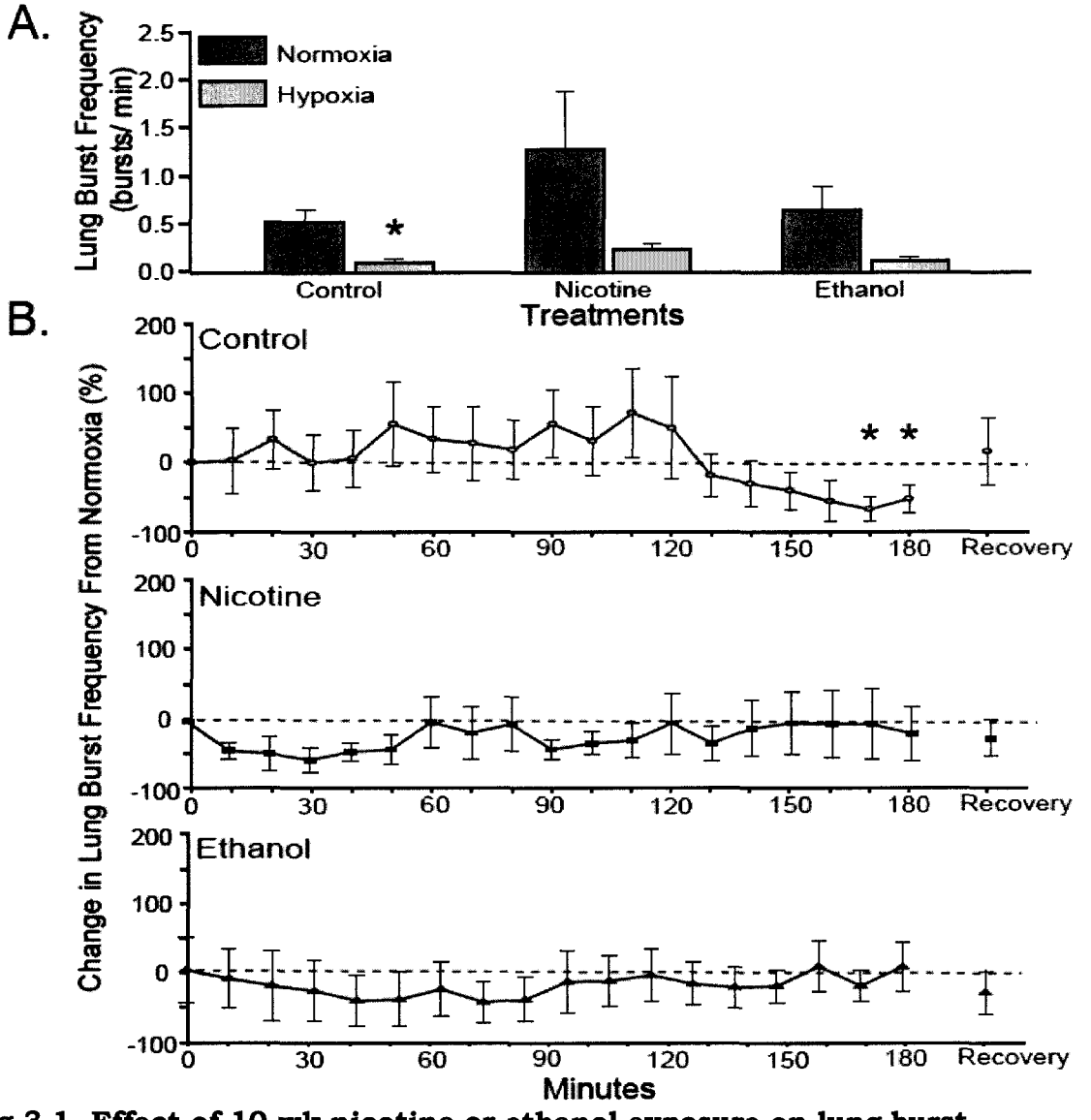


Fig 3.1 Effect of 10-wk nicotine or ethanol exposure on lung burst frequency of early metamorphic tadpoles. (A) Mean lung burst frequency during last 10 min of normoxia and peak response to hypoxia in control and nicotine- or ethanol-exposed early metamorphic tadpoles. (B) Percent change in lung burst frequency from normocapnia during each 10-min period of hypoxia. Control, but not nicotine- or ethanol-exposed, early metamorphic tadpoles decreased lung burst frequency in response to hypoxia (* = $P \leq 0.05$). Data presented are means ± SE for 6 - 7 tadpoles.

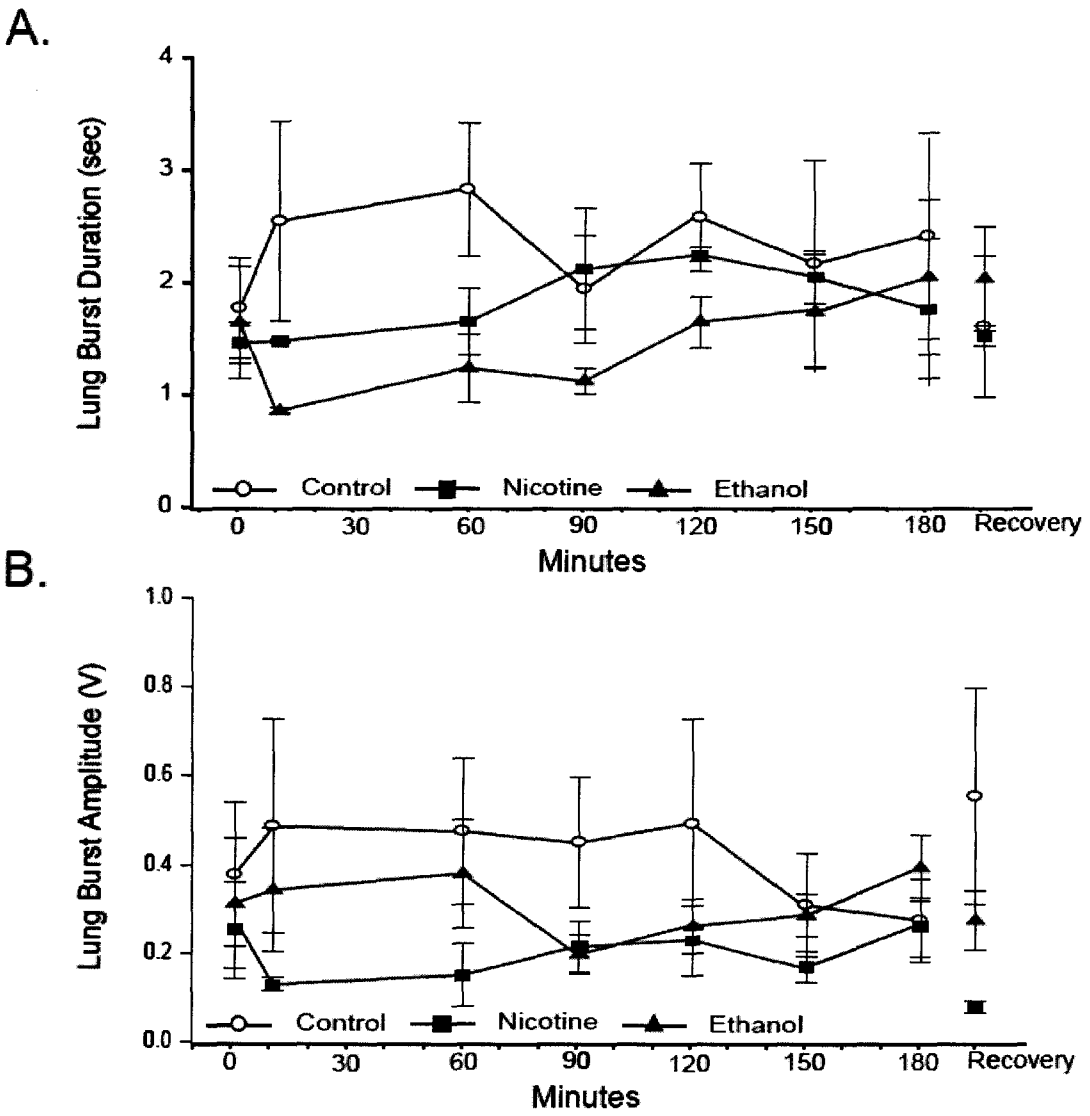


Fig 3.2 Effect of 10-wk nicotine or ethanol exposure on lung burst duration and amplitude of early metamorphic tadpoles. Mean lung burst duration (A) and amplitude (B) during last 3 min of normoxia,10, 60, 90, 120, 150, and 180 of min hypoxia, and 30 min of normoxic recovery in control and nicotine- or ethanol-exposed early metamorphic tadpoles. 10-wk nicotine or ethanol exposure had no effect on lung burst duration or amplitude. Hypoxia had no effect on lung burst duration or amplitude of any early metamorphic tadpole treatment group. Data presented are means ± SE for 6 - 7 tadpoles ($P > 0.05$).

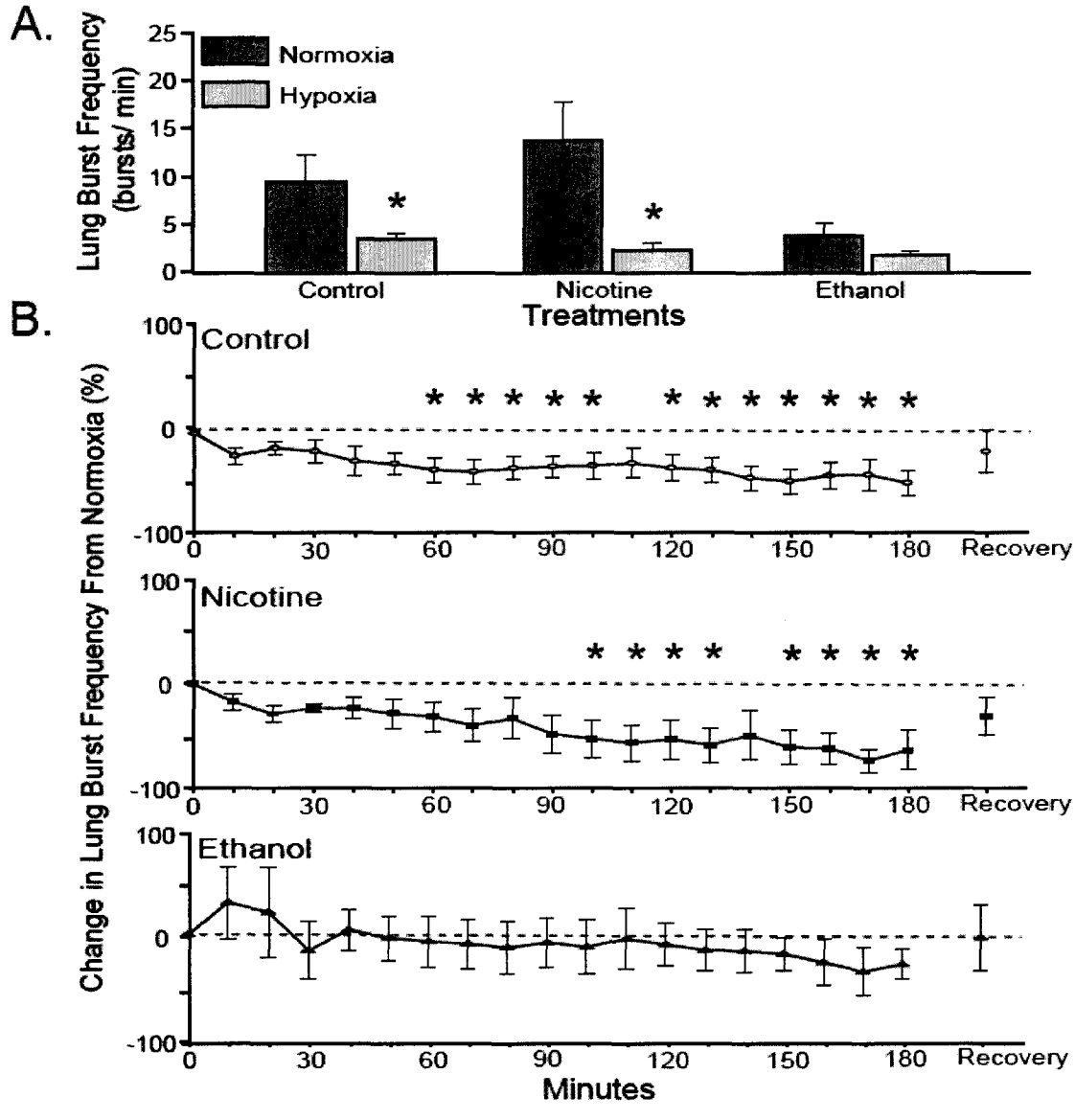


Fig 3.3 Effect of 10-wk nicotine or ethanol exposure on lung burst frequency of late metamorphic tadpoles. (A) Mean lung burst frequency during last 10 min of normoxia and peak response to hypoxia in control and nicotine- or ethanol-exposed late metamorphic tadpoles. (B) Percent change in lung burst frequency from normocapnia during each 10-min period of hypoxia. Control and nicotine-exposed tadpoles decreased lung burst frequency in response to hypoxia (* = $P \leq 0.05$). Data presented are means ± SE for 6 - 8 tadpoles.

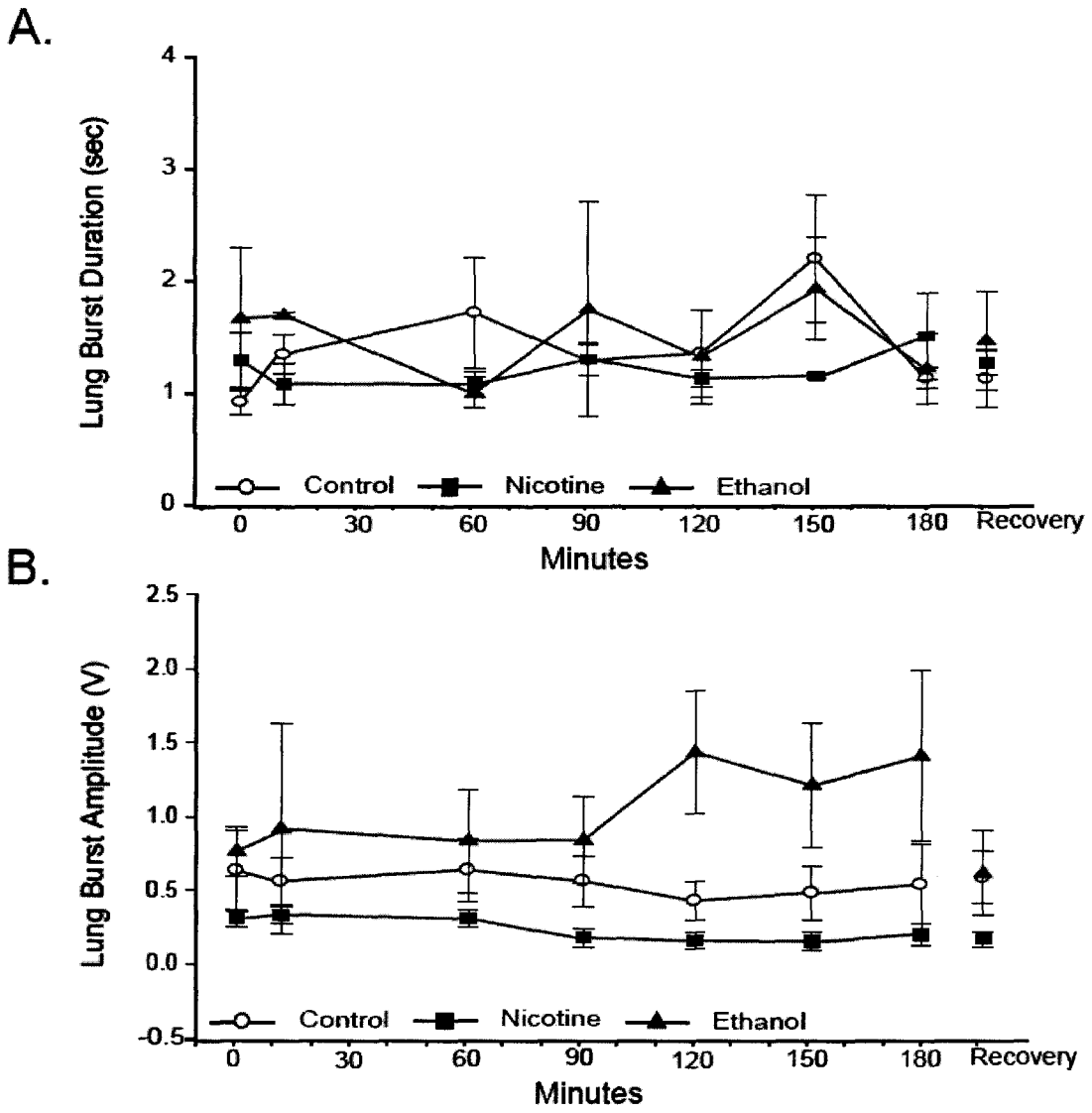
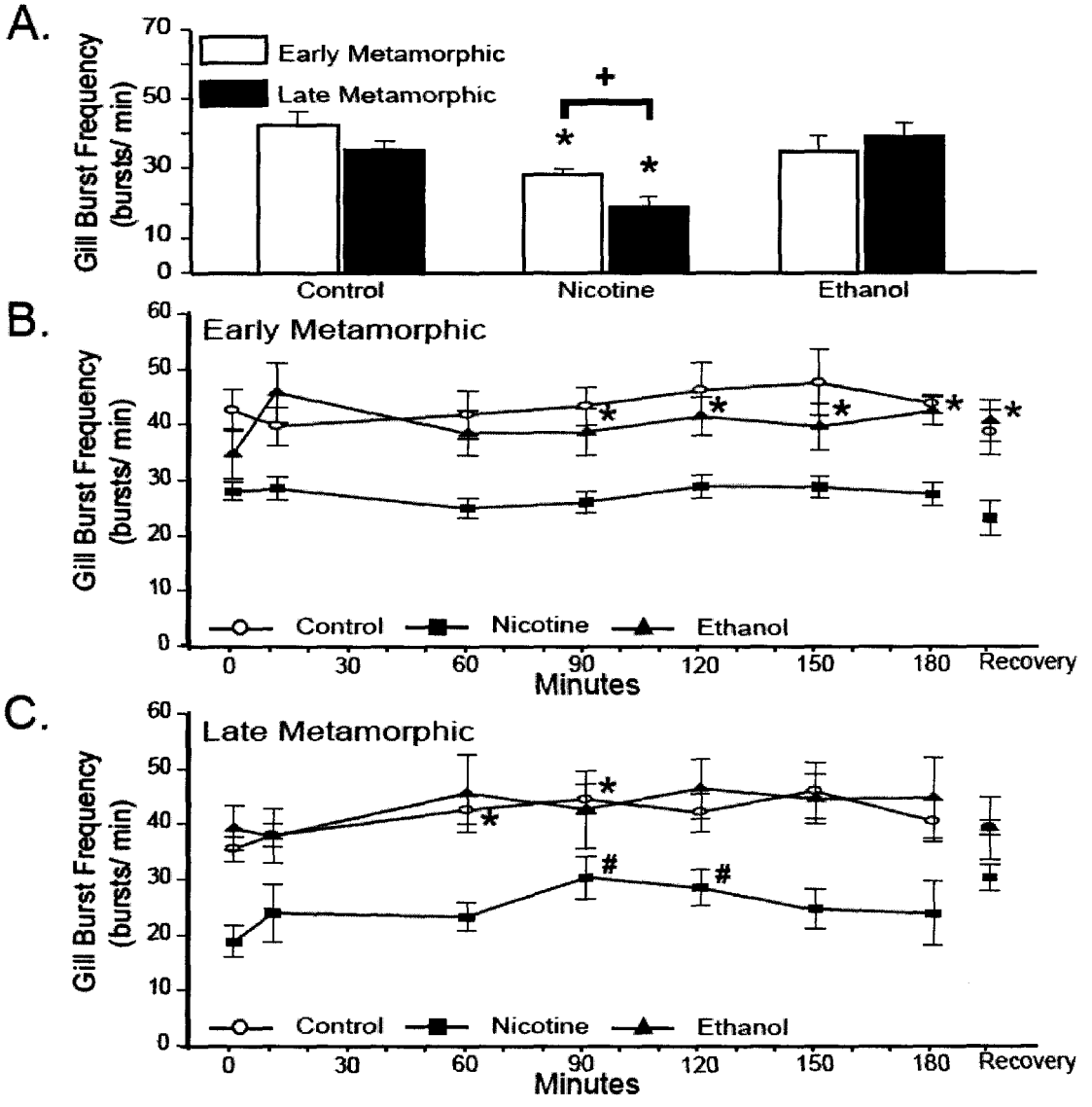


Fig 3.4 Effect of 10-wk nicotine or ethanol exposure on lung burst duration and amplitude of late metamorphic tadpoles. Mean lung burst duration (A) and amplitude (B) during last 3 min of normoxia,10, 60, 90, 120, 150, and 180 min of hypoxia, and 30 min of normoxic recovery in control and nicotine- or ethanol-exposed late metamorphic tadpoles. 10-wk nicotine or ethanol exposure had no effect on lung burst duration or amplitude. Hypoxia had no effect on lung burst duration or amplitude of any late metamorphic tadpole treatment group. Data presented are means ± SE for 6 - 8 tadpoles ($P > 0.05$).

Fig 3.5 Effect of 10-wk nicotine or ethanol exposure on tadpole gill burst frequency. (A) Mean gill burst frequency during last 3 min of normoxia in control and nicotine- or ethanol-exposed early and late metamorphic tadpoles. The gill burst frequency of early and late metamorphic nicotine-exposed tadpoles were both less than controls (* = $P \leq 0.05$). Late metamorphic nicotine-exposed tadpoles had a lower gill burst frequency than early metamorphic nicotine-exposed tadpoles (+ = $P = 0.014$). Mean lung burst frequency during last 3 min of normoxia, 10, 60, 90, 120, 150, and 180 min of hypoxia, and 30 min of normoxic recovery in control and nicotine- or ethanol-exposed early (B) and late metamorphic tadpoles (C). Early metamorphic ethanol-exposed tadpoles increased gill burst frequency from 90 -180 min of hypoxia (* = $P \leq 0.05$). Late metamorphic control tadpoles increased gill burst frequency at 60 and 90 min of hypoxia (* = $P \leq 0.05$). Late metamorphic nicotine-exposed tadpoles increased gill burst frequency at 90 and 120 min of hypoxia (# = $P \leq 0.05$). Data presented are means ± SE for 6 - 8 tadpoles.


A.
Gill Burst Frequency (bursts/ min)
Early Metamorphic
Late Metamorphic
Control
Nicotine
Ethanol
B.
Early Metamorphic
Gill Burst Frequency (bursts/ min)
Control
Nicotine
Ethanol
Minutes
Recovery
C.
Late Metamorphic
Gill Burst Frequency (bursts/ min)
Control
Nicotine
Ethanol
Minutes
Recovery

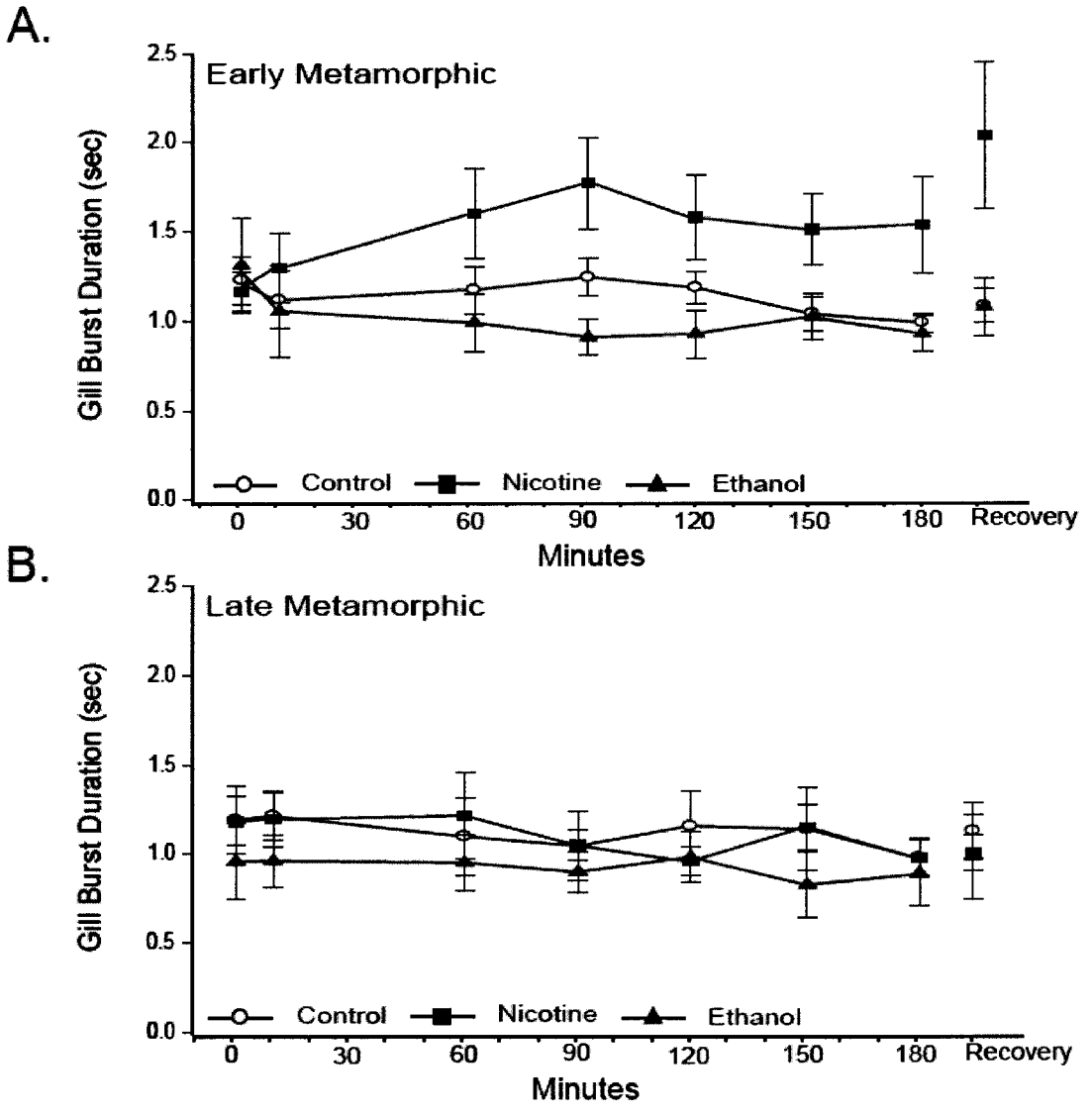


Fig 3.6 Effect of 10-wk nicotine or ethanol exposure on tadpole gill burst duration. Mean gill burst duration during last 3 min of normoxia,10, 60, 90, 120, 150, and 180 min of hypoxia, and 30 min of normoxic recovery in control and nicotine- or ethanol-exposed early (A) and late (B) metamorphic tadpoles. 10-wk nicotine or ethanol exposure had no effect on lung burst duration. Hypoxia had no effect on gill burst duration of any early or late metamorphic tadpole treatment group. Data presented are means ± SE for 6 - 8 tadpoles ($P > 0.05$).

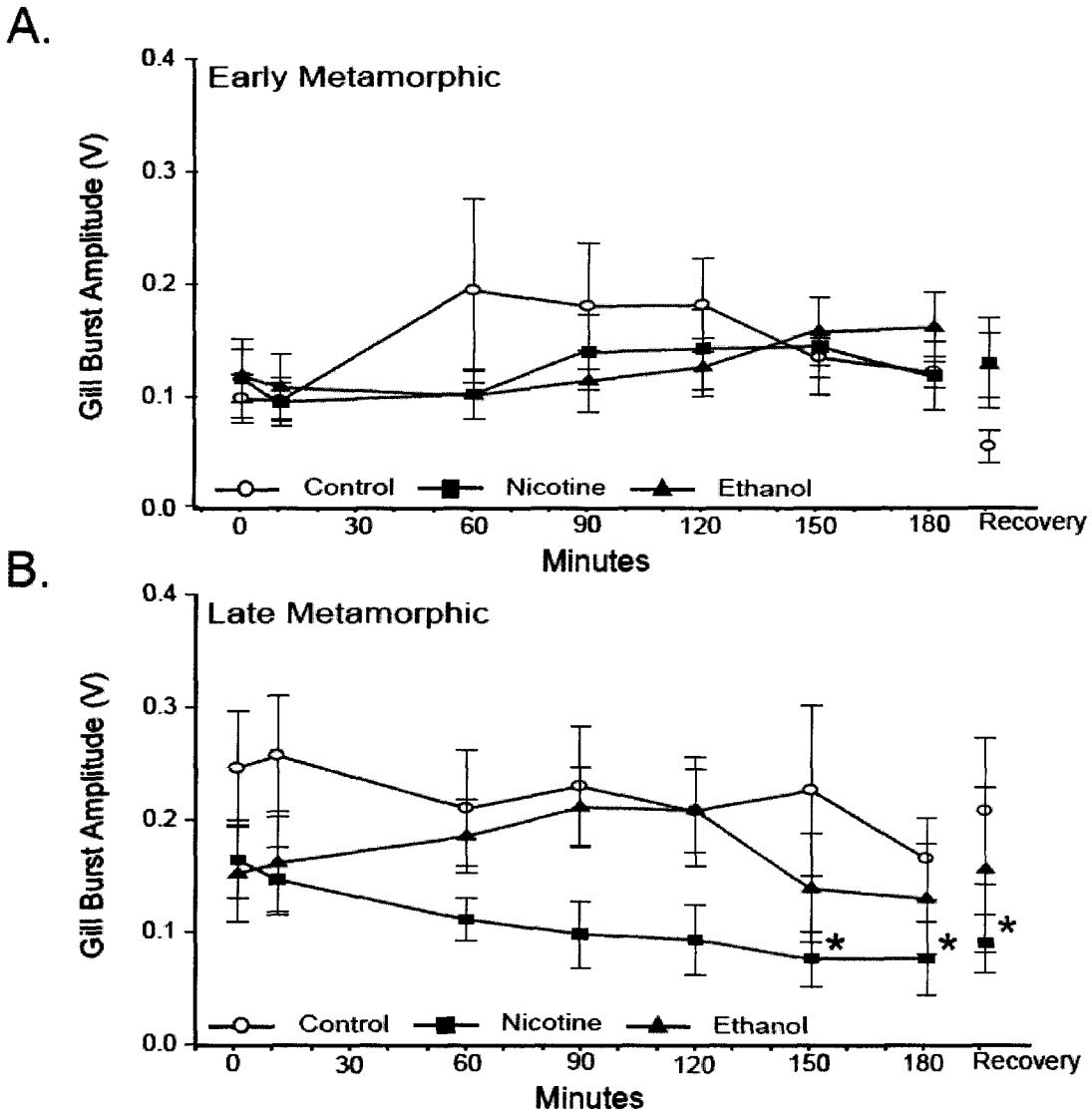


Fig 3.7 Effect of 10-wk nicotine or ethanol exposure on tadpole gill burst amplitude. Mean gill burst amplitude during last 3 min of normoxia,10, 60, 90, 120, 150, and 180 min of hypoxia, and 30 min of normoxic recovery in control, and nicotine- or ethanol-exposed early (A) and late (B) metamorphic tadpoles. 10-wk nicotine or ethanol exposure had no effect on normoxic gill burst amplitude. Hypoxia decreased gill burst amplitude of late metamorphic nicotine-exposed tadpoles at 150 and 180 min of hypoxia, and after 30 min of normoxic recovery (* = $P \leq 0.05$). Data presented are means ± SE for 6 - 8 tadpoles.

CHAPTER FOUR

Persistent loss and return of the hypercapnic neuroventilatory response following chronic nicotine or ethanol exposure[1]

4.1 Abstract

Neuroventilation is highly plastic; exposure to either of two distinct teratogens, nicotine or ethanol, during development results in a similar loss of the neuroventilatory response to hypercapnia in bullfrog tadpoles. Whether this functional deficit is permanent or transient following nicotine or ethanol exposure was unknown. Here we tested the persistence of impairments in the hypercapnic neuroventilatory response in tadpoles exposed to either 30 µg/L nicotine or 0.12 - 0.06 g/dL ethanol for 10 wk. Brainstem breathing-related neural activity was assessed in tadpoles were allowed to develop teratogen-free after either nicotine or ethanol exposure. Nicotine-exposed animals responded normally to hypercapnia after a 3 wk teratogen-free period, but the hypercapnic response in ethanol-exposed tadpoles remained

[1]Brundage, C.M. and Taylor, B.E. 2010. Persistent loss and return of the hypercapnic neuroventilatory response following chronic nicotine or ethanol exposure.(formatted for submission to) Developmental Neurobiology.

impaired. Tadpoles allowed to develop for only 1 wk after chronic nicotine exposure were unable to respond to hypercapnia. The hypercapnic response of ethanol-exposed tadpoles returned by 6 wk following chronic ethanol exposure. These findings suggest that some nicotine- and ethanol-induced functional impairments can be resolved during early development. Understanding both the disruptive effects of nicotine and ethanol exposure and how impaired responses return when teratogen exposure stops may offer insight into the function and plasticity of breathing control.

4.2 Introduction

Neuroventilation is considerably plastic, (Carroll, 2003; Mitchell and Johnson, 2003) and experiences generate short- to long-term changes in the function and morphology of the breathing control network (Bavis and Mitchell, 2008). The varying effects of these changes have a widespread impact on CO_2 homeostasis, because compensating for shifts in pH by offloading CO_2 is a critical homeostatic function of breathing (Milsom, 1995; Nattie, 1999; Putnam et al., 2004). Early exposure to either nicotine or ethanol during development results in a loss of the ventilatory response that normally counteracts elevated CO_2 (hypercapnia; Eugenin

et al., 2008; Taylor et al., 2008; see chapter two). This may explain why prenatal exposure to both nicotine and ethanol are risk factors for Sudden Infant Death Syndrome (SIDS; Iyasu et al., 2002; Kinney, 2009). An inability to adequately respond to hypercapnia has been hypothesized to contribute to SIDS (Shannon et al., 1977; Dunne et al., 1992; Richerson et al., 2001). Neuroplastic changes to the breathing control network that are evoked by nicotine or ethanol exposure result in impaired responses to hypercapnia in mice and bullfrog tadpoles (Eugenin et al., 2008; Taylor et al., 2008; Brundage and Taylor, 2009; see chapter two). Characterizing the neuroplastic changes induced by nicotine and ethanol could improve the understanding of the breathing control network and improve how clinical medicine addresses this vulnerability.

The timeline of impairments in hypercapnic responses offers insight into the development and lability of respiratory control. Response to hypercapnia is lost faster in early tadpole development following nicotine exposure compared to ethanol exposure (Brundage and Taylor, 2009; see chapter two). Despite the disparate actions of nicotine and ethanol in the brain, the breathing-related functional deficits following chronic exposures are the same. The duration of the impairment of hypercapnic

response may also differ following nicotine and ethanol exposure. The nicotine-induced impairment of hypercapnic response is transient in mice. Mice prenatally exposed to nicotine fail to respond to hypercapnia for 0 - 3 postnatal days; normal responses to hypercapnia return by postnatal day 8 (Eugenin et al., 2008). Persistence in the loss of hypercapnic responses following ethanol exposure, to our knowledge, has not been evaluated. It would be interesting if the timeline for recovery from nicotine and ethanol exposure were similar thereby suggesting that the neuroplastic changes evoked by nicotine and ethanol exposure may be comparable. Dissecting the mechanisms involved in nicotine and ethanol impairment of central hypercapnic drive can be aided significantly by first characterizing the developmental timeline for these impairments and their recovery.

The developing bullfrog tadpole has been used as a model to investigate the effects of nicotine and ethanol exposure on control of breathing (Taylor et al., 2008; Brundage and Taylor, 2009). The neuroventilation of bullfrogs can be quantified using an isolated brainstem preparation at all free-living developmental stages, and the exposure conditions of the tadpoles to nicotine and ethanol can be highly controlled due to their aquatic environment (Brundage and Taylor, 2009). Ten wk of chronic

nicotine or ethanol exposure results in the loss of the neuroventilatory response to hypercapnia in early metamorphic tadpoles (Taylor et al., 2008; Brundage and Taylor, 2009; see chapter two). We hypothesized that similar to that of mice, normal tadpole responses to hypercapnia would return if animals were allowed to live in a nicotine-free environment after their chronic nicotine exposure. Vulnerability to SIDS is resolved by the first year of life (Filiano and Kinney, 1994; Kinney, 2009), and we have found no reports of higher incidence among infants with fetal alcohol spectrum disorders. Thus, we also hypothesized that the effects of ethanol exposure would be resolved following an ethanol-free period. To our knowledge this is the first study to look at the persistence of any teratogen-induced impairment of neuroventilatory responses to hypercapnia in the tadpole, and the first to make such a consideration for any animal following ethanol exposure.

4.3 Materials and Methods

Animals

Studies were performed on *Lithobates* (formerly *Rana*) *catesbeiana* tadpoles (n = 34) purchased from a commercial supplier (Sullivan Co.

Inc., www.researchamphibians.com). Tadpoles were maintained at room temperature and were fed goldfish food daily. Tadpoles were housed for 10 wk in aquaria with dechlorinated water only, dechlorinated water containing nicotine (30 μg/L (–)-nicotine hydrogen tartrate salt; Sigma, www.sigmaaldrich.com), or dechlorinated water containing ethanol (0.12 - 0.06 g/dL). We chose 10-wk exposure because that duration of exposure impairs ventilatory responses to hypercapnia in both early and late stage tadpoles (Taylor et al., 2008; Brundage and Taylor, 2009; see chapter two). The concentration of nicotine was similar to that found in the body fluids of an average smoker (Moyer et al., 2002). The ethanol concentration varied due to the volatilization of ethanol from the tank and was equivalent to 0.75 - 1.5 times the 0.08 g/dL blood alcohol content that serves as the legal limit of many western countries (Caldeira et al., 2004). Following 10 wk of nicotine exposure, tadpoles were allowed to recover for either 1 wk (n = 6) or 3 wk (n = 8) in nicotine-free dechlorinated water. Ethanol-exposed tadpoles recovered for either 3 wk (n = 5) or 6 wk (n = 7).

Tadpole developmental stages were determined at the start of treatment and at the end of the 10-wk exposure to ensure developmental homogeneity. At the time of dissection each tadpole was at an early

stage of metamorphosis corresponding to developmental stages 7 - 15 in the classification scheme of Taylor and Köllros (1946). All animal care and experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Alaska Fairbanks and complied with all state and federal ethical guidelines.

Surgical preparation

Each tadpole was anesthetized by immersion for 1-2 min in cold (4 °C) 0.2 mM tricaine methanesulfonate (MS222; Sigma, www.sigmaaldrich.com) in dechlorinated water buffered to pH 7.8 with $NaHCO_3$. The front of the head rostral to the nares and the back of the body (hind limbs and tail, if present) were removed. The dorsal cranium and forebrain rostral to the diencephalon were resected and the fourth ventricle opened by removing the choroid plexus. The remaining brainstem and spinal cord were removed *en bloc* and further trimmed rostrally to the optic tectum and caudally at the brachial nerve. During dissection, exposed tissues were superfused with cold artificial cerebral spinal fluid (aCSF) composed of (in mM) 104 NaCl, 4 KCl, 1.4 $MgCl_2$, 10 D-glucose, 25 $NaHCO_3$ and 2.4 $CaCl_2$ equilibrated with 100 % O_2. These

methods have been used in our previous tadpole studies (Taylor et al., 2008; Davies et al., 2009).

The isolated brainstem was transferred to a 2.5 ml, Plexiglas, flow-through recording chamber and was supported, ventral side up, between coarse nylon mesh such that all surfaces were bathed with aCSF flowing from rostral to caudal at a rate of 5 ml/min. A supply of aCSF, equilibrated with O_2-CO_2 mixtures that produced the desired pH, flowed through plastic tubing to the recording chamber and bathed the isolated brainstem. The pH of the aCSF was maintained at either pH 7.8 (1.5 % CO_2: 98.5 % O_2; normocapnia) or pH 7.4 (5.0 % CO_2: 95.0 % O_2; hypercapnia) by adjusting the fractional concentrations of O_2 and CO_2 in the equilibration gas. CO_2 was monitored with a CO_2 analyzer (Capstar 100; CWE, www.cwe-inc.com). After isolation the brainstem was allowed to stabilize for 1 h while superfused at 23 °C, with aCSF of pH 7.8 (~9 torr P_{CO_2}).

Neurogram recording

Roots of the facial and hypoglossal nerves were drawn into glass suction electrodes pulled from 1-mm diameter capillary glass to tip diameters

that fit the nerve roots. Whole-nerve discharge was amplified (X100 by DAM 50 amplifiers, World Precision Instruments, www.wpiinc.com; X1000 by a four-channel model 1700 amplifier, A-M Systems, www.a-msystems.com) and filtered (100 Hz high pass to 1 kHz low pass). The amplified and filtered nerve output was sent to a data acquisition system (Powerlab, AD Instruments, www.adinstruments.com), which sampled at 1 kHz. The data were archived as whole-nerve discharge, and duplicate integrated (full-wave rectified and averaged over 200 ms) neurograms were acquired simultaneously. Such recordings were made during the initial 1 h post-isolation stabilization period and recorded continuously throughout the duration of each treatment protocol.

Data and statistical analyses

Neurograms recorded from the isolated tadpole brainstems were quantified for 30 min of normocapnia, 30 min of hypercapnia, and a subsequent 30 min return to normocapnia. Burst activity patterns were designated as either putative gill or putative lung breaths on the basis of the amplitude of the integrated nerve activity and the presence or absence of coincident firing in both the facial and hypoglossal nerves as previously described (Torgerson et al., 1998). Putative gill breaths had

lower integrated burst amplitude on the facial nerve than putative lung breaths and little or no coincident burst activity in the hypoglossal nerve. Putative lung breaths had higher integrated burst amplitude in the facial nerve and coincident burst activity in the hypoglossal nerve.

The frequencies of lung and gill ventilation were quantified as the number of bursts per minute for the last 3 min of normocapnia and hypercapnia. Taylor et al. (2003) demonstrated that an increase in lung burst frequency is the primary manifestation of the bullfrog hypercapnic response at all stages of their development (Fig. 4.1). Gill burst frequency during normocapnia, but not hypercapnia, was decreased after 10-wk exposure to nicotine but not ethanol (see chapter three). Therefore, gill neuroventilation during normocapnia in post-ethanol exposed tadpoles was not considered in this study. Percent changes in lung burst frequency during the last 3 min of hypercapnia were determined relative to the last 3 min of normocapnia. The mean values for each of the quantified burst frequencies were compared using repeated-measures analysis of variance (RM-ANOVA; SigmaStat, www.systat.com). When an RM-ANOVA indicated that significant differences existed, multiple comparisons were made using the Holm-Sidak multiple comparison test. Comparisons between control and treatment groups were conducted

using t-test comparisons (SigmaStat, www.systat.com). Values reported in the text are always means ± SE.

4.4 Results

Lung burst frequency after a 3-wk teratogen-free period following 10 wk of either nicotine or ethanol exposure are presented in Fig. 4.2. Control tadpoles increased lung burst frequency from 1.38 ± 0.36 bursts/min during normocapnia to 1.71 ± 0.51 bursts/min during hypercapnia (P = 0.029). Three wk of recovery following 10 wk of either nicotine or ethanol exposure did not significantly alter tadpole normocapnic lung burst frequencies (0.95 ± 0.30 bursts/min P = 0.393; 2.33 ± 0.48 bursts/min P = 0.138 for post-nicotine and post-ethanol exposure, respectively). Tadpoles previously exposed to nicotine for 10 wk responded similarly to control animals after 3 wk nicotine-free recovery by increasing lung burst frequency to 2.14 ± 0.41 bursts/min (P = 0.023); a 232.6 ± 0.5 % increase, compared to the 166.7 ± 85.9 % response to hypercapnia in controls. In contrast, 3-wk post-ethanol exposed tadpoles failed to increase lung burst frequency significantly during hypercapnia (2.46 ± 0.53 bursts/min; P = 0.987) demonstrating only a 2.8 ± 21.9 % change in lung burst frequency during hypercapnia. Thus, responses to

hypercapnia were still impaired following 3-wk recovery from chronic ethanol, but not nicotine, exposure.

The disparate responses following 3-wk post-exposure periods prompted interest to determine if any persistent impairment of the neuroventilatory hypercapnic response occurred in chronic nicotine-exposed tadpoles, because the previous studies (Taylor et al., 2008; Brundage and Taylor, 2009) had quantified this central hypercapnic response immediately after chronic exposure, without any nicotine-free period. 10-wk nicotine-exposed tadpoles were, therefore, allowed to recover for 1 wk in nicotine-free water before experimentation. The normocapnic lung burst frequency of 1-wk post-nicotine exposed tadpoles was slightly, although not significantly, elevated compared to controls (2.53 ± 0.41 bursts/min $P = 0.059$; Fig. 4.3). Responses to hypercapnia, however, were blocked; in fact there was a modest reduction in lung burst frequency during hypercapnia (1.56 ± 0.49 bursts/min; $P = 0.287$), a -43.2 ± 9.5 % change in lung burst frequency. This reduction in lung burst frequency was not significant nor was there any significant change in lung burst frequency after 30-min return to normocapnia (1.39 ± 0.62 bursts/min). Therefore, the hypercapnia-induced increase in lung burst frequency that was impaired following 10-wk nicotine exposure remained impaired 1 wk

following nicotine exposure, but was fully returned after 3 wk in a nicotine-free environment.

Our previous work identified a diminished normocapnic gill burst frequency in tadpoles immediately following 10-wk nicotine exposure (see chapter three). Here gill burst frequency was quantified during normocapnia in both the 1-wk and 3-wk post-nicotine exposed tadpoles (Fig. 4.4). One wk after exposure the gill burst frequency was significantly elevated compared to control tadpoles (36.2 ± 2.8 and 46.9 ± 2.7 bursts/min; $P = 0.02$ for control and 1-wk post-nicotine exposed tadpoles, respectively). Gill burst frequency of 3-wk post-nicotine exposed tadpoles was not significantly different from controls (41.3 ± 4.4 bursts/min; $P = 0.343$), nor was it significantly different from 1-wk post-nicotine exposed tadpoles ($P = 0.342$). Thus, although lung burst responses to hypercapnia continued to be impaired after a 1-wk nicotine-free period, the decreased gill burst frequency induced by chronic nicotine exposure was replaced with a full recovery and facilitation by 1-wk post-nicotine exposure.

The persistent impairment of the hypercapnic neuroventilatory response in 3-wk post-ethanol exposed tadpoles prompted interest to determine if

the reduction in hypercapnic ventilatory drive was permanent following chronic ethanol exposure. Tadpoles allowed to recover for 6 wk after 10-wk ethanol exposure had a normocapnic lung burst frequency that was not significantly different than controls (0.57 ± 0.10 bursts/min; P = 0.065; Fig. 4.5). Six-wk post-ethanol exposed tadpoles did increase lung burst frequency in response to hypercapnia (1.05 ± 0.22 bursts/min; P = 0.036). This response to hypercapnia was reduced, but was not significantly different from that of controls (86.9 ± 38.9 %; P = 0.497). Lung burst frequency fully returned to pre-hypercapnic levels by the end of the normocapnic recovery period (0.49 ± 0.14 bursts/min). Thus, impairments in the hypercapnic response of chronic ethanol-exposed tadpoles persisted for 3 wk, but not 6 wk after ethanol exposure was stopped.

4.5 Discussion

It took between 1 and 3 wk for the tadpole hypercapnic neuroventilatory response to return following 10 wk of chronic nicotine exposure. This is considerably more rapid than the 3- to 6-wk recovery period required following 10 wk of chronic ethanol exposure. Thus, we have demonstrated that, as in mice in which the central hypercapnic response

is transiently impaired by developmental nicotine exposure (Eugenin et al., 2008), nicotine- and ethanol-induced impairments of the tadpole central hypercapnic response are transient. It is noteworthy that, just as there is a difference in the duration of chronic exposure required to induce an impairment (Brundage and Taylor 2009; see chapter two), nicotine and ethanol differ in the duration that their impairments persist after teratogen exposure is stopped. The mechanisms that underlie the differences in these two teratogens, regarding both the time to impairment and time to recovery are of particular interest.

Persistent impairment of the tadpole central hypercapnic response that results from chronic nicotine or ethanol exposure may be an example of developmental neuroplasticity. Developmental neuroplasticity has been defined as a long-term change induced by experiences during a critical period of development and has recently been recognized as an important phenomenon in respiratory control (Carroll, 2003; Bavis and Mitchell, 2008). The term developmental neuroplasticity may be more appropriately applied to the effect of chronic nicotine exposure, because early metamorphic tadpoles have a increased vulnerability to nicotine exposure compared to later developmental stages (Brundage and Taylor, 2009, Brundage et al., 2010). Chronic ethanol exposure, although not

evaluated in adult bullfrogs, causes similar impairment of the central hypercapnic responses during both early and late periods of tadpole metamorphosis (see chapter two). Nonetheless, chronic exposure to nicotine or ethanol during tadpole development resulted in a deficit that lasted for 1-6 wk after exposure, and in the case of nicotine, similar exposure after metamorphosis did not induce a similar impairment in the central hypercapnic response (Brundage et al., 2010). Thus, we have identified two paradigms of persistent change in the respiratory control network induced by environmental factors experienced during tadpole development. We believe these research paradigms can now be applied to investigate the mechanisms underlying nicotine- and ethanol-induced developmental neuroplasticity of the breathing control network, a developmental neuroplasticity that is deleterious and may be shared broadly among vertebrates.

The mechanisms of neural change that underlie this plasticity, which manifest as a loss of central hypercapnic ventilatory responses, may result from persistent nicotinic acetylcholine receptor stimulation by nicotine or the potentiation of GABAergic signaling in the case of ethanol (Aguayo et al., 2002; Slotkin et al., 2002; Breese et al., 2006). Chronic stimulation may result in desensitization to subsequent

acetylcholine/nicotine, GABA, or other neurotransmitter systems reportedly altered by chronic nicotine and ethanol exposure (Allan et al., 1998; Covernton and Lester, 2002; Fregosi and Pilarski, 2008; Dopico and Lovinger, 2009; Duncan et al., 2009). Nicotinic and GABA receptors are involved in neurogenesis (Zahalka et al., 1992; Represa and Ben-Ari, 2005; Dwyer et al., 2008). The persistent loss of hypercapnic responses may not be limited to cell signaling but may also reflect altered neurodevelopment, a change in the types or numbers of cells participating in the breathing control network. Investigations of the mechanisms underlying nicotine- and ethanol-induced developmental neuroplasticity of the breathing control network are warranted and should be pursued on both the cellular and receptor levels to identify the specific modes of impairment in these animal models.

When nicotine and ethanol were removed from the tadpole environment, there was a subsequent change in neural control of breathing, a central ventilatory response to high CO_2 returned. Thus, we have identified another type of neuroplasticity in breathing control, one that is beneficial but not necessarily developmental in nature. Identifying the mechanisms contributing to the return of the central hypercapnic response will provide important information about neuroplasticity in the breathing

control network. Although the response to hypercapnia returned, it is unclear if the original mechanisms providing that response recovered or if a new mechanism, one that accommodated the deleterious effects of nicotine and ethanol, arose and facilitated a functional central hypercapnic response. Whether through the recovery of an original mechanism or the rise of a new mechanism, a change in the neural network that controls breathing was induced after nicotine and ethanol exposure were stopped. The change in signaling of the central hypercapnic response, as another instance of neuroplasticity, is expected to have resulted from neurogenesis, synaptogenesis, or changes in synaptic composition, and any or all of these factors have the potential to contribute to the renewed manifestation of the central hypercapnic response (Bavis and Mitchell, 2008; Dwyer et al., 2008).

Immediately following 10 wk of chronic nicotine exposure the normocapnic gill burst frequency in isolated tadpole brainstems was decreased (see chapter three). After 1 wk post-nicotine exposure gill burst frequency during normocapnia was greater in brainstems isolated from post-exposure tadpoles than controls, although not significantly greater than the frequencies reported in the previous study (see chapter three). Here gill burst facilitation returned to control levels by 3-wk post-

nicotine exposure. Thus, with respect to gill burst activity during normocapnia, chronic nicotine exposure induced an initial decrease and recovery from nicotine exposure induced an over-corrective increase followed by a return to baseline levels. This pattern of change in response to changes in nicotine exposure may be consistent with a nicotine-induced desensitization of nicotinic acetylcholine receptors that is compensated by an increase in receptor density, and a further change in receptors when the desensitization is rectified. Such alteration in receptor density have been reported for cortical neurons and is termed homeostatic neuroplasticity (Turrigiano, 1999; Desai, 2003; Wierenga et al., 2006; Aoki et al., 2009), which is an adaptation to maintain signal strength in the face of receptor desensitization. There is no direct evidence to support that a change in receptors occurred in the present study; rather we raise this issue to emphasize that the mechanism underlying this phenomenon is potentially of interest. The return to a functional central hypercapnic response was not the only neuroplastic change resulting from the cessation of chronic nicotine exposure, the baseline frequency of gill neuroventilation was potentially affected as well.

Developmental consistency exists in the hypercapnic response; CO_2 induces an increase in lung burst frequency early in metamorphosis and this response is maintained across ontogeny (see chapter two). Although the actual frequency of hypercapnic lung bursting increases with development, the hypercapnia-induced percent change in lung busrting is relatively constant (Taylor et al., 2003; see chapter two). Thus, although the normocapnic neuroventilation varied between treatment groups the response to hypercapnia should have been consistent. The major reductions in the percent change in ventilation during in hypercapnia in the 1-wk post-nicotine exposed and 3-wk post-ethanol exposed groups suggest these responses were impaired and not the result of intra-group variability.

In this study we used a tadpole model to investigate development of neural control of breathing, and we saw that the SIDS risk factors of chronic developmental exposure to nicotine (Milerad and Sundell, 1993; Adgent, 2006) and ethanol (Iyasu et al., 2002; Kinney, 2009) impaired central response to a ventilatory stressor, hypercapnia. These impairments persisted after exposure to the teratogens stopped, but the central hypercapnic response was eventually recovered, as it is in mice (Eugenin et al., 2008) after prenatal nicotine exposure. Characterizing

the mechanistic consequences of chronic nicotine and ethanol exposure, the persistence of these consequences, as well as the mechanisms that support the return of functionality, may offer significant insight into neuroplasticity of the breathing control network and its hypercapnic response, which may extend the understanding of the pathogenesis of SIDS.

4.6 Acknowledgments

Dr. Barbara E. Taylor served as my advisor and was the principal investigator for the laboratory overseeing this research. This work was supported by NIH-NINDS grant #U54 NS041069-06A1.

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4.8 Figures

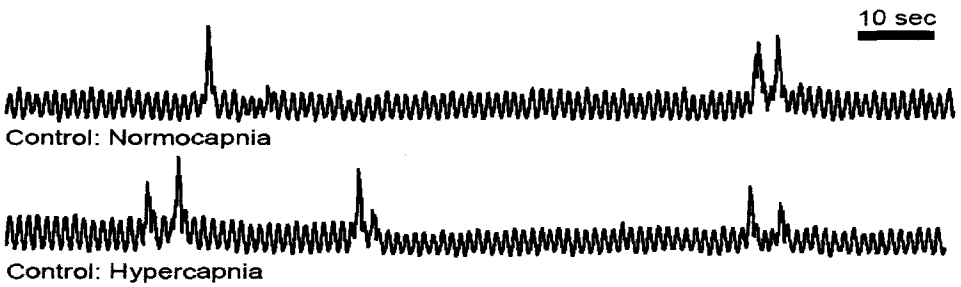

Fig 4.1 Lung burst frequency increased in response to hypercapnia. Representative integrated neurograms recorded over 2 min from the facial nerve root of a control bullfrog tadpole during normocapnia and hypercapnia. Control tadpoles increased the number of lung burst per minute in response to hypercapnia.

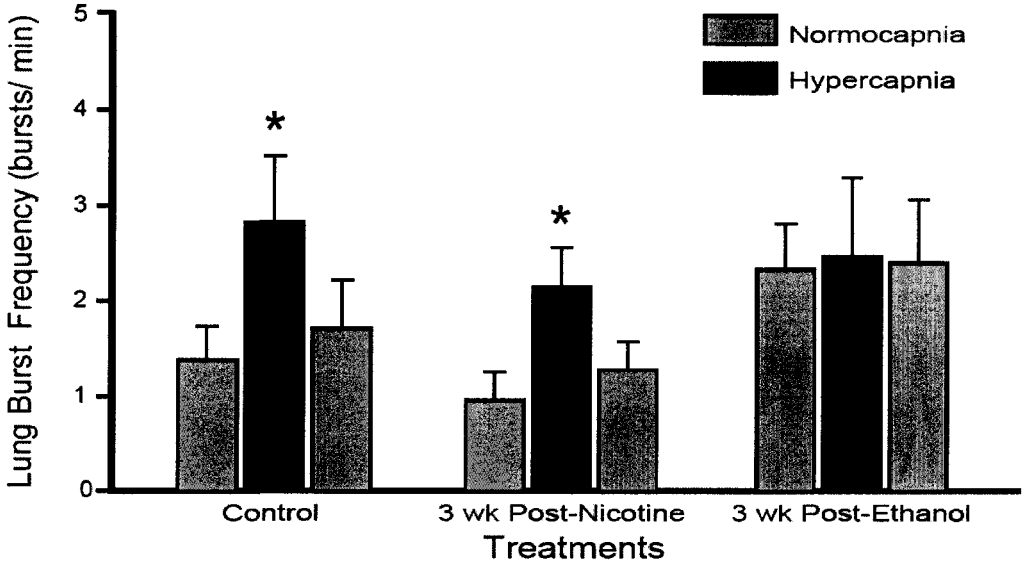

Fig 4.2 Central hypercapnic response returned following 3-wk recovery in nicotine- but not ethanol-exposed tadpoles. Mean lung bursts per min over the last 3 min of normocapnia, hypercapnia and return to normocapnia. Control and 3-wk post-nicotine exposed tadpoles responded significantly to hypercapnia (* = $P < 0.05$). 3-wk post-ethanol exposed tadpoles did not respond to hypercapnia ($P > 0.05$). Data presented are means ± SE for 6 - 8 tadpoles.

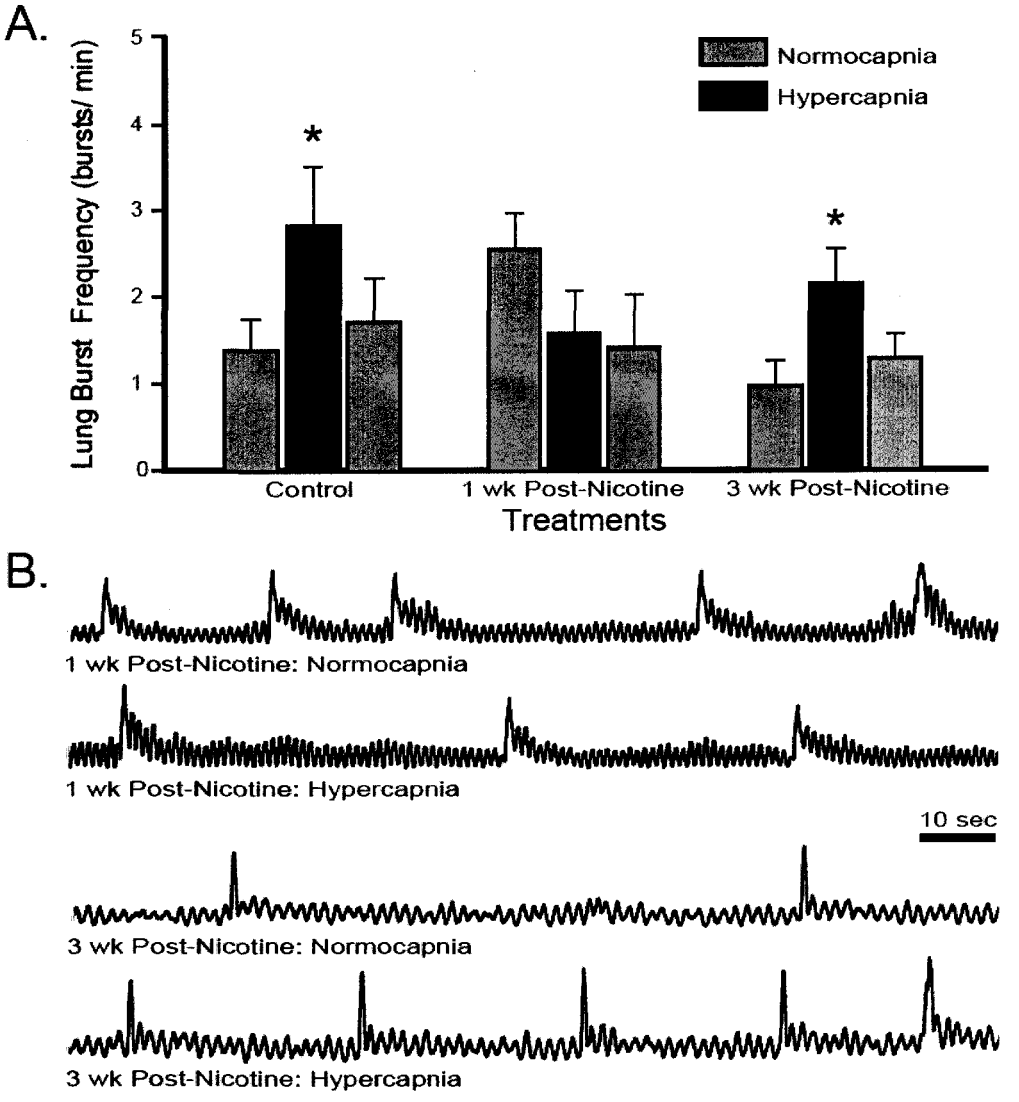

Fig 4.3 Central hypercapnic response was impaired 1-wk post-nicotine but returned by 3-wk post-nicotine exposure. (A) Mean lung bursts per min over the last 3 min of normocapnia, hypercapnia, and return to normocapnia. 1-wk post-nicotine exposed tadpoles did not respond to hypercapnia. 3-wk post-nicotine exposed tadpoles increased lung burst frequency during hypercapnia (* = $P < 0.05$). Data presented are means ± SE for 6 - 8 tadpoles. (B) Representative integrated neurograms recorded over 2 min from the facial nerve root during normocapnia and hypercapnia.

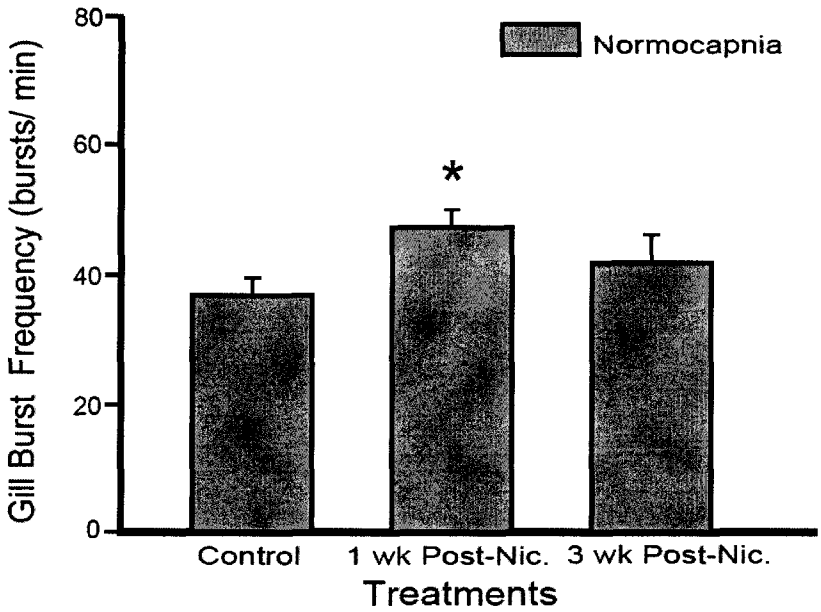

Fig 4.4 Gill burst frequency was elevated 1-wk post-nicotine but not 3-wk post-nicotine exposure. Mean gill bursts per min over the last 3 min of normocapnia for control, 1-wk, and 3-wk post-nicotine exposed tadpoles. 1-wk post-nicotine exposed tadpoles had an elevated gill burst frequency compared to controls (* = $P < 0.05$). The gill burst frequency of 3-wk post-nicotine exposed tadpoles was similar to control preparations. Data presented are means ± SE for 6 - 8 tadpoles.

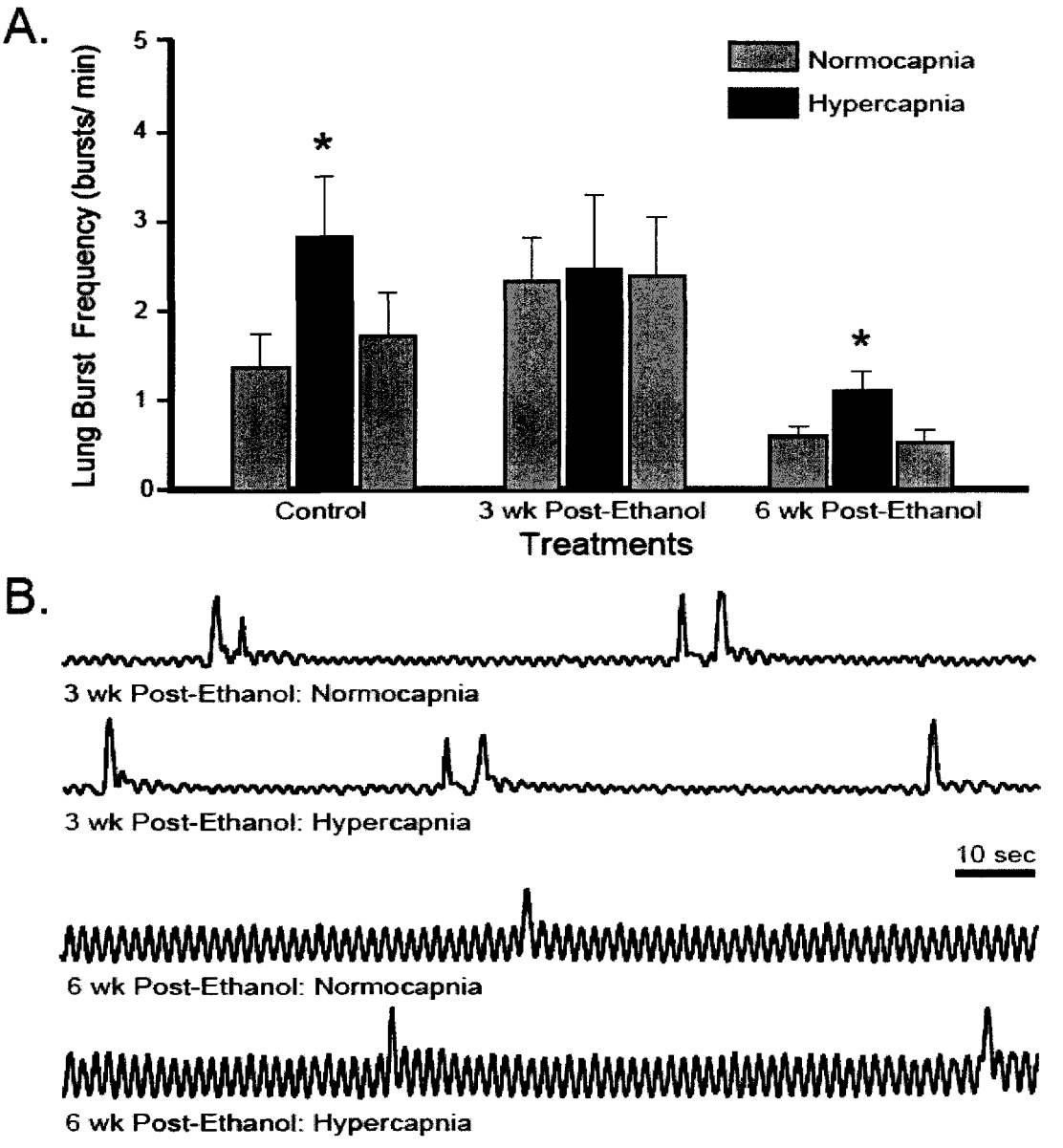

Fig 4.5 Central hypercapnic response was lost 3-wk post-ethanol but returned by 6-wk post-ethanol exposure. (A) Mean lung bursts per min over the last 3 min of normocapnia, hypercapnia, and return to normocapnia. (3-wk post-ethanol exposed tadpoles did not respond to hypercapnia. Control and 6-wk post-ethanol exposed tadpoles increased lung burst frequency during hypercapnia (* = $P < 0.05$). Data presented are means ± SE for 6 - 8 tadpoles. (B) Representative integrated neurograms recorded over 2 min from the facial nerve root during normocapnia and hypercapnia.

CHAPTER FIVE

General conclusions

5.1 Neuroventilatory effects of nicotine and ethanol on the developing bullfrog

Ten-wk but not 3-wk exposure to 0.12 - 0.06 g/dL ethanol impaired bullfrog tadpole central responses to hypercapnia (see chapter two), a drive to increase breathing to off load CO_2 (Taylor et al., 2003a, Taylor et al., 2003b). This functional impairment was seen whether exposure took place early or late in tadpole development (see chapter two). Early metamorphic tadpoles regained central responses to hypercapnia if they were allowed to develop for 3 - 6 wk post-ethanol exposure. This was longer than the 1 - 3 wk required to regain the central hypercapnic response in early metamorphic tadpoles exposed to 30 μg/L nicotine for 10 wk (see chapter four).

Normocapnic/normoxic neuroventilation was unaffected by chronic ethanol exposure (see chapter two and three). Early but not late metamorphic tadpole brainstem preparations exposed acutely to 0.08

g/dL ethanol did have a reduced normocapnic lung burst frequency, but responded normally to hypercapnia (see chapter two). Exposure to 0.12 - 0.06 g/dL ethanol for 10 wk also impaired central response to hypoxia, which manifests as a reduction in breathing activity to match reduced demand for O_2 (see chapter three). Similar to the impaired central hypercapnic response in these animals, the impaired hypoxic response was found in tadpoles exposed to ethanol either early or late in metamorphosis (see chapter three). Unlike 10-wk ethanol-exposure, early but not late metamorphic 10-wk nicotine-exposed tadpoles demonstrated impaired central hypoxic responses (see chapter three). Late metamorphic tadpoles exhibited a central hypoxic response, but it occurred later in the hypoxic treatment (see chapter three). Ten wk of nicotine exposure also altered normocapnic/normoxic neuroventilation; gill burst frequency of both early and late metamorphic tadpoles was reduced relative to control tadpoles (see chapter three). Late metamorphic tadpoles had a significantly lower gill burst frequency following 10-wk nicotine exposure than early metamorphic tadpoles (see chapter three). One-wk post-nicotine exposure, the gill burst frequency of early metamorphic tadpoles increased to levels greater than control tadpoles and returned to control normocapnic/normoxic levels by 3-wk post-nicotine exposure (see chapter four).

The findings just outlined from the previous chapters may be considered together with those of similar studies from our laboratory. These studies determined the central hypercapnic response of early metamorphic tadpoles could be impaired following either 3 wk or 10 wk of exposure to 30 μg/L nicotine (Brundage and Taylor, 2009). Three-wk nicotine exposure had no effect, however, on the central hypercapnic responses of late metamorphic tadpoles or juvenile bullfrogs (Brundage and Taylor, 2009, Brundage et al., 2010b). Ten-wk nicotine exposure did impair the central hypercapnic response of late metamorphic tadpoles (Taylor et al., 2008, Brundage and Taylor, 2009). The brainstems of juvenile bullfrogs and late metamorphic tadpoles were more susceptible to the effects of acute nicotine exposure (Brundage et al., 2010a). Nicotine (18 μg/L) applied directly via the bath superfusing the brainstem decreased the lung burst frequency of late metamorphic tadpoles and juvenile bullfrogs and attenuated the central hypercapnic response in the juvenile bullfrogs (Brundage et al., 2010a). The effect of acute nicotine exposure of brainstems isolated from juvenile bullfrogs could be reversed with 1 nM mecamylamine, a noncompetitive nicotinic acetylcholine receptor (nAchR) antagonist (Brundage et al., 2010a). The effect of nicotine could be mimicked in the juvenile bullfrog with application of 1 μM galantamine,

an acetylcholinesterase inhibitor (Brundage et al., 2010b). Acute exposure to 18 μg/L nicotine had no effect on early metamorphic tadpoles (Brundage et al., 2010a). The central hypercapnic response in early and late metamorphic tadpole preparations was reduced with higher concentrations of nicotine (100 and 50 μg/L nicotine for early and late metamorphic tadpoles, respectively; Brundage et al., 2010a).

Considered collectively these studies produce two independent models for the study of neuroplasticity, neurotoxicology, and the development of central breathing control. The first is the nicotine-exposure model. In this model the early metamorphic tadpoles are more vulnerable to chronic nicotine exposure. Central hypercapnic responses of early metamorphic tadpoles are lost following only 3 wk of nicotine exposure, but late metamorphic tadpoles require 10 wk for impairment to be created (Brundage and Taylor, 2009). Central hypoxic responses are lost following 10-wk exposure in early metamorphic tadpoles, but the response in 10-wk nicotine-exposed late metamorphic tadpoles are consistent (although delayed) with controls (see chapter three). This model is distinct from the ethanol-exposure model, which shows no developmental change in teratogen vulnerability. Ten wk but not 3 wk of ethanol exposure impairs the central hypercapnic response of early and

late metamorphic tadpoles (see chapter two). Ten wk of ethanol exposure impairs the central hypoxic response throughout tadpole metamorphosis (see chapter three).

5.2 Mechanistic insight from teratogen exposure

The differences in the teratogen-exposure models I have developed suggest some of the mechanisms involved in central ventilatory responses to hypercapnia and hypoxia. The developmental increase in sensitivity to acute nicotine suggests that the role of nicotinic acetylcholine receptors (nAchRs) in breathing control changes during development (Brundage and Taylor, 2009). Acute nicotine and galantamine reduced lung burst activity and attenuated responses to hypercapnia (Brundage et al., 2010a, Brundage et al., 2010b). The role of nAchRs may thus be to regulate breathing activity and either turn off or restrict central responses to hypercapnia. Chronic exposure to nicotine also restricted central responses to hypercapnia, but with an inverse developmental relationship to that of acute nicotine (Brundage et al., 2010a). One possible explanation is that chronic nicotine exposure sensitized lung-related neural activity to nicotine. Thus, in the absence of

exogenous nicotine the endogenous acetylcholine provided enough activation of nAchRs to limit activity during hypercapnia.

Gill-related neuroventilation in nicotine-exposed tadpoles showed classic desensitization. Chronic exposure resulted in reduced gill burst frequency, but 1-wk post-nicotine exposure the burst activity was augmented and only returned to control levels after 3-wk post-nicotine exposure (see chapter three and four). Gill and lung ventilation are interdependent. It is possible that the action of nicotine in one area had multiple effects that directly or indirectly altered other burst parameters. Nicotine, for example, has been found to increase GABA release, GABAergic currents, and $GABA_A$ receptor expression with chronic exposure (Luo et al., 2004). An increase in nAchRs may have resulted in the decrease of breathing activity through the up-regulation of GABAergic inhibition, and not through sensitization to nicotine.

Ethanol exposure has many effects on the central nervous system. The most noted is the action of ethanol as a positive allosteric modulator of $GABA_A$ receptors (Olsen et al., 2007). Both acute ethanol and acute GABA exposure on the tadpole brainstem decrease normocapnic/normoxic lung burst activity (Galante et al., 1996, Broch et

al., 2002; see chapter two). The central hypoxic response in tadpoles is dependent on GABA signaling, and chronic exposure to ethanol blocks that response in early and late metamorphic tadpoles (Fournier et al., 2007; see chapter three). This suggests that chronic ethanol causes desensitization to ethanol and/or damages key components of the response mechanisms to hypoxia and hypercapnia.

Discussion of GABA is recurrent in this dissertation; chronic exposure to both nicotine and ethanol have been found to alter either GABAergic signaling or GABA receptor subtype expression (Luo et al., 2004, Miura et al., 2006, Luo et al., 2007, Lobo and Harris, 2008, Isayama et al., 2009). GABAergic processes may be involved in generating responses to hypercapnia and hypoxia (Curran et al., 2001, Gourine and Spyer, 2001, Zhang et al., 2003, Hsieh et al., 2004, Fournier et al., 2007), and GABA receptors may be altered in the brains of SIDS infants (Broadbelt et al., 2009). Preliminary experiments in our laboratory have shown that chronic ethanol exposure caused desensitization to acute GABA, ethanol, and the $GABA_A$ receptor agonist muscimol (Brundage et al. 2008, Iceman et al. 2008). GABA is not, however, the only neurotransmitter involved in ethanol's mediation of ventilatory responses. Acute GABA caused a decrease in both lung and gill burst activity (Broch et al., 2002). Acute

ethanol exposure, in contrast, reduced lung activity, but left very robust, even augmented, gill burst activity (see chapter two). Ethanol's action through non-GABA associated mechanisms seems likely to be responsible for the retention of gill activity.

The weight of scientific evidence supports the involvement of serotonin in responses to hypercapnia and hypoxia (Richerson et al., 2001, Penatti et al., 2006, Corcoran et al., 2009, Dergacheva et al., 2009). SIDS infants have been found to share abnormalities in the serotonergic system of the brainstem (Kinney et al., 2003, Paterson et al., 2006, Kinney et al., 2009). Preliminary results from our laboratory point to similar abnormalities in serotonergic neuron and receptor subtype expression in early and late metamorphic tadpoles exposed to either ethanol or nicotine (Audie et al. 2008). The signaling systems affected, either directly or indirectly, by nicotine or ethanol exposure appear to overlap, this may be responsible for their shared neuroventilatory impairments.

Clearly understanding the mechanisms by which nicotine and ethanol impair responses to hypercapnia and hypoxia is the next critical aim. That aim would not be possible without first characterizing the functional impairment and the conditions involved in its expression.

Understanding the effects of nicotine and ethanol on the developing nervous system and its networks for controlling rhythmic behavior, such as breathing, is applicable for many reasons, not the least of which is SIDS.

The pathogenesis of SIDS may comprise failures in the brainstem networks that modulate cardiac rhythms and control breathing rhythms (Hunt, 1992, Duncan et al., 2009). Early developmental exposure to nicotine and ethanol are risk factors for SIDS, and the chronic nicotine and ethanol exposure models described in this dissertation resulted in impairments of the breathing control network (see chapter two and three). Accordingly, the research presented here may have established a research model in which neural deficits and their causative mechanisms can be investigated and may provide insight into some SIDS cases. Alternatively, SIDS may result from another failed process, one outside the cardiorespiratory system and not yet identified. To accommodate any physiological stressor, an organism must adapt to and/or compensate for a physiological change. My research models demonstrate that chronic exposure to both nicotine and ethanol can result in an impaired adaptive response to the physiological stressor, hypoxia (see chapter three), and an impaired compensatory response to the physiological stressor,

hypercapnia (Taylor et al., 2008, Brundage and Taylor, 2009; see chapter two). Regardless of how directly applicable these models are to the pathogenesis of SIDS, at the least, they establish a means for investigating the deleterious effects that chronic exposure to nicotine or ethanol have on signaling within neural networks.

5.3 Trends in tadpole responses to ventilatory stressors

When I started my dissertation research not much was understood about the temporal aspects of bullfrog tadpole responses to the ventilatory stressors hypercapnia and hypoxia. Before investigating the neuroventilatory effects of nicotine or ethanol exposure I had to first characterize temporal changes in the lung burst frequency increase in bullfrog responses to hypercapnia (Taylor et al., 2003a, Taylor et al., 2003b). Early metamorphic tadpoles breathe infrequently (Burggren and West, 1982). Quantifying changes in lung burst frequencies over short periods of time is difficult because the values are low. For example, an animal increasing breathing activity from .25 to .33 lung bursts per minute requires analysis of at least 4 min of neurogram recordings. As a result of the difficulty associated with quantifying tadpole lung burst

frequencies, early studies proposed that early metamorphic tadpoles did not respond to hypercapnia (Torgerson et al., 1997).

I quantified lung burst frequency for every minute of normocapnia and hypercapnia in every preparation. My results supported the finding that tadpole hypercapnic responses are consistent across development (Taylor et al., 2003a, Taylor et al., 2003b). I determined that tadpole brainstem preparations could take over 45 min to return to normocapnic lung burst frequency levels after hypercapnia (Brundage et al., 2010a). I also determined that responses to multiple 30-min hypercapnic challenges in the same preparation were equivalent (Brundage et al., 2010a). These observations provided valuable insight for my research on neuroteratogen treatments, but they are also of value to any investigation on the neuropharamcology of tadpole central hypercapnic responses.

Bullfrog central hypoxic responses have not been as extensively evaluated as those for hypercapnia, but my research helps shed light on inconsistencies reported elsewhere in the literature. The hypoxic response appears to change with bullfrog ontogeny. The ventilatory depression characteristic of the central hypoxic response was seen late in hypoxia for early metamorphic tadpoles and earlier for late metamorphic

tadpoles (Winmill et al., 2005; see chapter three). This suggests the hypoxic response develop early in tadpole metamorphosis. An increase in sensitivity and/or a decrease in tolerance leads to a shorter reaction time to hypoxia. These findings are consistent with the Winmill and colleagues (2005) study that looked at the long-term effects of severe hypoxia, but differ from a study by Fournier and colleagues (2007) who looked at responses to hypoxia 10 min into the hypoxia treatment. Fournier and colleagues identified a significant increase in lung burst activity in early metamorphic tadpoles 10 min into hypoxia. We quantified lung burst frequencies for the entire hypoxic treatment. Early metamorphic tadpole lung burst activity at the beginning of hypoxia was highly variable. That variability may explain why responses at 10 min differed between studies (see chapter three). A reduction in lung burst frequency with sustained hypoxia is the consistent response in all studies.

Bullfrog responses to hypoxia are reported to be heavily dependent on GABA (Fournier et al., 2007), which is supported by the major action of chronic ethanol on central responses to hypoxia (see chapter three). Chronic nicotine treatment affected only early metamorphic tadpole brainstem responses to hypoxia (see chapter three). There may be a change in the role of nAchRs in hypoxic ventilatory responses,

neurodevelopment, or some combinatorial effect of chronic nicotine exposure that I have not yet considered.

The bullfrog central hypercapnic response is consistent across development from early metamorphic tadpoles to adults (Taylor et al., 2003a, Taylor et al., 2003b; see chapter two and four). Therefore, the central response to hypercapnia was already developed at the onset of our chronic nicotine and ethanol treatments, and our chronic treatments disrupted an already functional network rather than an emerging network. This finding may partially account for ethanol exposure ablating the tadpole central hypercapnic response regardless of the developmental timing of exposure (see chapter two). In contrast, nicotine disrupts bullfrog neuroventilatory responses to hypercapnia in a developmental stage-dependent manner (Brundage and Taylor, 2009, Brundage et al., 2010a). The role of nAchRs in bullfrog control of breathing is unclear. We determined that acute nicotine can decrease central lung burst activity and response to hypercapnia, as well as alter breathing patterns (Brundage et al., 2010a). The effects of nicotine exposure suggest that nAchRs play a role in regulating baseline breathing activity and/or responses to hypercapnia.

It is interesting that responses to hypercapnia produce the same functional response at all stages of bullfrog development, but the responses to hypoxia change, perhaps even become more sensitive, with bullfrog ontogeny. The major drive to breathe in aquatic vertebrates is the drive to acquire O_2 (Milsom, 2008). Here I report that tadpoles don't exhibit a central response to hypoxia until late in the hypoxic treatment (see chapter three). Studies *in vivo* do show an increase in gill activity with hypoxia, this must be peripherally mediated (West and Burggren, 1982). Data suggest the development of the central hypoxic response is a maturation of adaptive ventilatory down-regulation, which seems to be concomitant with the developing bullfrog becoming more dependent on aerial respiration. Tetrapods are challenged less by the need to acquire oxygen than the need to off load CO_2 (Milsom, 1995, 2002, 2008). Hypercapnic drive is not a major factor in aquatic ventilation, yet early metamorphic tadpoles have a fully functional central hypercapnic response (Taylor et al., 2003a, Taylor et al., 2003b, see chapter two). It is possible that central CO_2 chemosensitivity forms concurrently with lung rhythm generation. It may be important to note that although the degree of impairments from nicotine or ethanol may be similar in some cases between early and late metamorphic tadpoles, the physiological consequences of those impairments may differ depending on

development, the degree to which the bullfrog favors aquatic or terrestrial habitats and how reliant the animal is on gills and/or lungs for breathing. The central hypercapnic response is not, however, essential for breathing, nicotine or ethanol knock out responses to hypercapnia and animals continue to breathe (Taylor et al., 2008, Brundage and Taylor, 2009; see chapter two).

5.4 Neuroplasticty and functional recuperation

At the cellular, inter-cellular, and network level, neurological systems are incredibly plastic (Carroll, 2003, Turrigiano and Nelson, 2004, Cheron et al., 2008). Perturbations at each level elicit widespread reactions; that can cause long-term alterations in the central nervous system and its functions (Bavis and Mitchell, 2008). These alterations include changes in the composition of neural populations, which are brought about by changes in neurogenesis and neural apoptposis, as well as changes in cell signaling, which are brought about by changes in synaptogenesis and synaptic pruning and/or by changes in neurotransmitter and receptor expression. Induced alterations likely produce a combination of several types of changes, and the cumulative effect can be a maintenance of function, homeostatic neuroplasitcity (Desai, 2003, Turrigiano and

Nelson, 2004, Ishikawa et al., 2009); an alteration in function that is limited to a critical window of development, developmental neuroplasticity (Carroll, 2003, Bavis and Mitchell, 2008), or a reinstatement of function, recuperative neuroplasticity (see chapter four).

Equally as important as identifying the timeline, conditions, and mechanisms that generate functional impairments following nicotine and ethanol exposure, an example of neuroplasticity because they represent neural change, is identifying the persistence of these impairments and the timeline, conditions, and mechanisms that underlie recuperative neuroplasticity (see chapter four). Return of a functional central hypercapnic response post-teratogen exposure is a clear example of recuperative neuroplasticity. This response returned in post-teratogen exposed tadpoles either through a recovery of the original mechanism that produced the response or development of a new mechanism that accommodated changes in the original mechanisms and generated a functional response despite them (see chapter four). Changes from sub-cellular to system-wide levels may be involved in the loss and return of the hypercapnic response in the bullfrog after nicotine and ethanol exposure. I have developed tadpole models of teratogen-induced

impairment of the breathing control network, and I have characterized the timing of the neuroplasticity that underlies impairment and recovery of a critical network function; therefore, characterization of these neuroplasticities is a reachable goal.

As research tools, the utility of these tadpole models of nicotine- and ethanol-induced neural impairments increased exponentially when I demonstrated their recuperative neuroplasticity. The tadpole models developed in the course of my dissertation research will be useful for investigations of the mechanisms that underlie central ventilatory responses to hypercapnia and hypoxia, the mechanisms of neural signaling that are vulnerable to nicotine and ethanol, and the mechanisms of several types of neuroplasticity. The appropriate starting point for these investigations will be to compare the phenotypic heterogeneity within breathing control networks of functionally normal tadpoles prior to teratogen treatment with those functionally impaired after teratogen treatment, and with those that have regained normal function after teratogen treatment has stopped. Information gathered from these and subsequent investigations will have broad implications for the treatment and prevention of nicotine- and ethanol-related

pathologies and may lead to treatments for breathing-related disorders like SIDS.

5.5 Significance of research

The effects of nicotine and ethanol exposure on development of the control of breathing have only recently become a research focus. The first study to my knowledge to quantify impairments in the central hypercapnic responses following either developmental nicotine or ethanol exposure used tadpoles (Taylor et al., 2008). Including work presented in this dissertation, five other papers have investigated the effects of these teratogens on neural control of breathing, four were conducted at the University of Alaska Fairbanks (UAF) using bullfrogs (Brundage and Taylor, 2009, Brundage et al. 2010b; see chapters two and four), and another was conducted using mice (Eugenin et al., 2008). To date the only experiments that have addressed ethanol-induced effects on the central hypercapnic response during development were conducted on tadpoles at UAF (Taylor et al., 2008; see chapter two). My research goal was not to develop an alternative model or to validate known information, but rather to characterize a model and produce some of the first studies on the effects of teratogens on central chemosensitivity.

Establishing the first link between chronic developmental exposure to nicotine or ethanol and impairment of the central hypercapnic response is significant in and of itself. Those findings serve as a foundation of my research. I attempted to go further and understand the developmental and temporal factors that influenced the teratogen-induced impairments. As a result these studies are the first to look at the effect of development and exposure duration on central hypercapnic response impairments, and they are the first to emphasize the importance of recovery from these impairments (Brundage and Taylor, 2009; see chapters two and four).

Responses to hypoxia following nicotine and ethanol exposure have been more extensively studied; however, most of these studies have looked at initial, compensatory, and peripheral responses to hypoxia (Slotkin et al., 1995, Bamford et al., 1996, Hafstrom et al., 2002, Neff et al., 2004, Dubois et al., 2008). Our studies on teratogen-induced impairment of central hypoxic responses are the first to investigate the effects of ethanol exposure on the central hypoxic response (see chapter three), and one of only two to do so after nicotine exposure (Simakajornboon et al., 2004 see chapter three).

It is widely accepted that nicotine and ethanol are deleterious to development. The significance of my research lies in providing a tool to help researchers understand the mechanisms by which nicotine and ethanol evoke their deleterious effects and the natural process that can return a neural network to normal functionality. The implications of this work may benefit not only SIDS research, but research on neural network impairments in general.

5.6 References

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APPENDIX

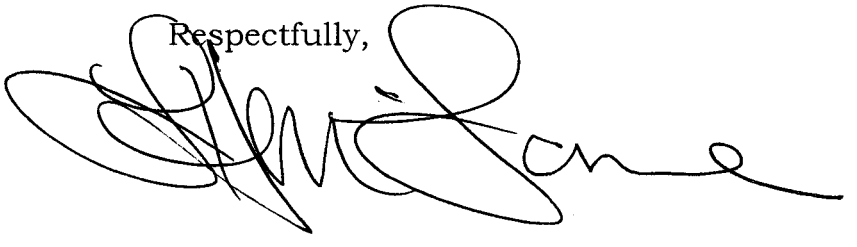
Permission to use manuscript contribution in dissertation

April 14, 2010

To whom it may concern:

I, Lisa H. McLane, am fully aware that my contribution to the manuscript "Chronic nicotine and ethanol exposure both disrupt central ventilatory responses to hypoxia in bullfrog tadpoles" will be included in a chapter of Cord M. Brundage's PhD Thesis.

Respectfully,



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